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Awanwee PETCHKONGKAEW

Pour obtenir

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REDUCTION DU NIVEAU DE MYCOTOXINES DANS LA FERMENTATION DU SOJA

Soutenue le 11 avril 2008, devant le Jury composé de:

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REDUCTION OF MYCOTOXIN CONTAMINATION LEVEL DURING SOYBEAN FERMENTATION

Awanwee Petchkongkaew

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Food Technology

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นางสาวอวันวี เพชรคงแก้ว

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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy

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การศึกษาวิจัยในครั้งนี้เกี่ยวข้องกับการลดระดับปริมาณสารพิษจากเชื้อราในระหว่างกระบวนการหมักถั่วเน่าซึ่งเป็นผลิตภัณฑ์ที่นิยมบริโภคกันมากในแถบภาคเหนือของประเทศไทย นอกจากนี้ยังทำการศึกษาวิจัยเกี่ยวกับการจำแนกและบ่งชี้ลักษณะของเชื้อราที่มีความสามารถในการสร้างสารพิษโอคราทอกซินเอซึ่งปนเปื้อนในองุ่นที่ได้ทำการเพาะปลูกในไร่องุ่นจำนวน 7 แห่งในประเทศฝรั่งเศสอีกด้วย ผลการทดลองในงานวิจัยส่วนนี้พบว่า แอสเปอร์จิลลัส คาร์บอนาเรียส และ แอสเปอร์จิลลัส ไนเจอร์ เป็นเชื้อราที่ปนเปื้อนมากในองุ่นและมีความสามารถในการสร้างสารพิษโอคราทอกซินเอสูงที่สุด รองลงมาคือเชื้อรา แอสเปอร์จิลลัส จาโปนิคัส

สำหรับงานวิจัยที่เกี่ยวข้องกับการลดระดับการปนเปื้อนของปริมาณสารพิษจากเชื้อราในระหว่างกระบวนการหมักถั่วเน่านั้นพบว่าเชื้อบาซิลลัสจำนวน 23 ไอโซเลทได้ถูกคัดเลือกจากผลิตภัณฑ์ถั่วเน่า โดยเชื้อบาซิลลัสนับว่าเป็นจุลินทรีย์ที่มีบทบาทมากในการผลิตผลิตภัณฑ์ชนิดนี้ โดยเชื้อบาซิลลัสจะทำหน้าที่เป็นกล้าเชื้อทำให้ผลิตภัณฑ์ที่ได้มีคุณภาพสม่ำเสมอตามต้องการ ในขณะเดียวกันจากรายงานการวิจัยต่างๆพบว่าเชื้อบาซิลลัสบางสายพันธุ์มีความสามารถในการลดปริมาณการปนเปื้อนของสารพิ

ษ ที่ ส ร้ า ง ขึ้ น จ า ก เ ชื้ อ ร า อ ย่ า ง เ ช่ น อ ะ ฟ ล า ท อ ก ซิ น แ ล ะ โ อ ค ร า ท อ ก ซิ น เ อ ดั ง นั้ น วั ต ถุ ป ร ะ ส ง ค์ ข อ ง ง า น วิ จั ย นี้ คื อ เพื่อศึกษาถึงความสามารถของเชื้อบาซิลลัสในการยับยั้งการเจริญเติบโตของเชื้อราแอสเปอร์จิลลัส ฟลาวัสและแอสเปอร์จิลลัส เ ว อ ร์ เ ท อ ร์ ดิ จ เ กี ย ร์ เ อ็ น อ า ร์ อ า ร์ แ อ ล 3174 และความสามารถในการกำจัดสารพิษที่สร้างขึ้นจากเชื้อราทั้งสองชนิดนี้ ผลการทดลองพบว่า เชื้อบาซิลลัส ซี เอ็ม 21 มีความสามารถในการยับยั้งการเจริญเติบโตของเชื้อราทั้งสองชนิด น อ ก จ า ก นี้ ยั ง พ บ อี ก ว่ า เ ชื้ อ บ า ซิ ล ลั ส ดังกล่าวมีความสามารถในการกำจัดสารพิษที่สร้างขึ้นจากเชื้อรา ซึ่งก็คือ อะฟลาทอกซิน บีวัน และโอคราทอกซิน เอ โดยความสามารถในการกำจัดสารพิษดังกล่าวเท่ากับ 74 เปอร์เซ็นต์และ 92.5 เปอร์เซ็นต์ตามลำดับ นอกจากนี้ยังพบอีกว่าเชื้อบาซิลลัส เอ็ม เฮช เอส 13 ก็ มี ค ว า ม ส า ม า ร ถ ดั ง ก ล่ า ว เ ช่ น กั น โดยสามารถกำจัดสารพิษอะฟลาทอกซิน บีวันได้ถึง 85 เปอร์เซ็นต์และยังสามารถยับยั้งการเจริญเติบโตของเชื้อราในสกุล แ อ ส เ ป อ ร์ จิ ล ลั ส ไ ด้ อี ก ด้ ว ย โ ด ย ก ล ไ ก ใ น ก า ร กำ จั ด สา ร พิ ษ อ ะ ฟ ล า ท อ ก ซิ น บี วั น โดยเชื้อบาซิลลัสนี้นั้นยังไม่เป็นที่ทราบแน่ชัดแต่อาจเกี่ยวข้องกับการดูดซับสารพิษที่ผนังเซลล์ของจุลินทรีย์หรือไม่ก็เกี่ยวข้อง

กับกระบวนการแอมโมนิเอชั่น ซึ่งจุลินทรีย์ชนิดนี้มีความสามารถในการสร้างแอมโมเนียได้สูงถึง 220 มิลลิกรัมต่อลิตร ในขณะที่กลไกในการกำจัดสารพิษโอคราทอกซิน เอ มีความเป็นไปได้ว่าจะเกี่ยวข้องกับเอนไซม์คาร์บอกซีเปปติเดส เอ โดยเอนไซม์ดังกล่าวนี้จะไปทำการเปลี่ยนสารพิษโอคราทอกซิน เอ ไปเป็นกรดอะมิโนที่มีชื่อว่า ฟีนิลอะลานีน และโอคราทอกซินอัลฟ่า ซึ่งสารตัวนี้มีความเป็นพิษน้อยกว่าโอคราทอกซินเอถึงหนึ่งพันเท่า

จากผลการทดลองดังกล่าวเราจะพบว่าเชื้อบาซิลลัสทั้งสองสายพันธุ์นี้มีความสามารถในการยับยั้งการเจริญเติบโตของเชื้อราในสกุลแอสเปอร์จิลลัสและนอกจากนี้ยังมีความสามารถในการกำจัดสารพิษที่สร้างขึ้นจากเชื้อราดังกล่าวอีกด้วย ทั้งนี้เอนไซม์ที่ได้จากเชื้อบาซิลลัสกลุ่มนี้ยังคงมีประโยชน์ต่อแวดวงอุตสาหกรรมอาหารในแง่ของการกำจัดหรือลดปริมาณสารพิษอะฟลาทอกซิน บี วัน และโอคราทอกซิน เอ สุดท้ายนี้ผลิตภัณฑ์ถั่วเน่าที่นิยมบริโภคกันเป็นส่วนมากทางภาคเหนือของประเทศไทยน่าจะมีความปลอดภัยต่อผู้บริโภค

สาขาวิชาเทคโนโลยีอาหาร ลายมือชื่อนักศึกษา ___
_____ ปีการศึกษา 2550
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ลายมือชื่ออาจารย์ที่ปรึกษาร่วม __
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ลายมือชื่ออาจารย์ที่ปรึกษาร่วม __
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AWANWEE PETCHKONGKAEW: **REDUCTION OF MYCOTOXIN CONTAMINATION LEVEL DURING SOYBEAN FERMENTATION**. THESIS ADVISOR: ASST.PROF.DR. PIYAWAN GASALUCK, Ph.D. 212 PP. ISBN xxx-xxx-xxx-x

Aflatoxin/ Ochratoxin A/ Biodegradation/ *Bacillus* Spp./ *Aspergillus*/ Thua-Nao.

This thesis deals with the reduction of mycotoxin contamination level during soybean fermentation (Thua-Nao).

Beside this work, isolation, characterization, and ochratoxin A production ability of toxigenic fungi from French grapes were also study. Results of this latter part showed that *Aspergillus carbonarius* and *Aspergillus niger* are the most ochratoxin A producer in wine grape from France. Furthermore, *Aspergillus japonicus* can produce a little bit quantity of ochratoxin A in wine grape too.

Regarding to the main part of the work, 23 isolates of *Bacillus* spp. were isolated from Thai Thua-Nao. An *Aspergillus flavus* aflatoxin producing strain was also isolated from Thua-Nao whereas an *Aspergillus westerdijkiae* was chosen as an OTA producing reference strain. The objectives were to find an efficient *Bacillus* strain for:

- Growth inhibition of *Aspergillus flavus* and *Aspergillus westerdijkiae* NRRL 3174.

- Limitation of aflatoxin B_1 production.

- Mycotoxins, aflatoxin B_1 and ochratoxin A detoxification.

Among the results, *Bacillus* CM 21, which was identified later by ITS sequencing as *Bacillus licheniformis*, showed the highest ability on inhibition of growth of both *Aspergillus* strains and both of mycotoxins removal (decrease of 74% of AFB_1 and 92.5% of OTA). Another *Bacillus* strain, MHS 13, inhibiting both *Aspergillus* growth and detoxifying 85% of AFB_1 was identified as *Bacillus subtilis*.

Finally, culture supernatant and cellular extract from both interested *Bacillus* strains were tested for aflatoxin B_1 and ochratoxin A degradation ability in order to know their degradation mechanisms. Moreover, study on optimal condition for aflatoxin B_1 and ochratoxin A degradation were also conducted. All results indicated that OTA was significantly degraded by culture supernatant from *Bacillus licheniformis* CM 21 ($p<0.0001$) in OTalpha. The percentage of OTA degradation was 97.5% and the optimal activity of its culture supernatant was found at pH 7.0 and 37°C with 24 h culture incubation time and 2 h contact time. Moreover, OTA was also significantly degraded by culture supernatant from *Bacillus subtilis* MHS 13 ($p<0.0017$) at pH 5.0 and 37°C with 48 h culture incubation time and 2 h contact time. The proposed degradation mechanism should be extracellular and carboxypeptidase A probably responsible for this degradation since no activity was found for the intracellular extract. However, AFB_1 could be degraded by neither culture supernatant

nor cellular extract from both of these microorganisms. Hence, the AFB_1 detoxification mechanism may be due to non-enzymatic mechanism.

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AWANWEE PETCHKONGKAEW: **REDUCTION DU NIVEAU DE MYCOTOXINES DANS LA FERMENTATION DU SOJA.** DIRECTEUR DE THESE : PROF.DR. PATRICIA TAILLANDIER, Ph.D. 212 PP. ISBN xxx-xxx-xxx-

Aflatoxin/ Ochratoxin A/ Biodegradation/ Bacillus Spp./ Aspergillus/ Thua-Nao.

Cette étude traite de la réduction du niveau de contamination en mycotoxines pendant la fermentation du soja (produit alimentaire thaïlandais appelé Thua-Nao).

En marge de ce travail, l'isolement et la caractérisation de la capacité à produire de l'ochratoxine A a été évaluée chez des champignons toxinogènes isolés de raisins français destinés à la vinification. Les résultats de cette partie ont montré que *Aspergillus carbonarius* et *Aspergillus niger* sont les champignons les plus producteurs en France. Cependant, *Aspergillus japonicus* peut aussi être à l'origine de faibles quantités d'ochratoxine A au niveau des raisins.

En ce qui concerne la principale partie de ce travail, 23 isolats de *Bacillus* spp. ont été obtenus à partir de Thua-Nao thaïlandais. Une souche d'*Aspergillus flavus* productrice d'aflatoxine a aussi été isolée de Thua-Nao tandis qu'une souche d'*Aspergillus westerdijkiae* (ex *ochraceus*) a été choisie comme référence

d'*Aspergillus* producteur d'OTA. Les objectifs étaient de trouver des souches de *Bacillus* efficaces pour:

- inhiber la croissance d'*Aspergillus flavus* et *Aspergillus westerdijkiae* NRRL 3174.
- limiter la production d'aflatoxine B_1.
- détoxifier le milieu en diminuant la teneur en mycotoxines : aflatoxine B_1 (AFB_1) et Ochratoxine A (OTA).

Parmi les résultats principaux, *Bacillus* CM 21, ensuite identifiée comme *Bacillus licheniformis* par séquençage des ITS, a montré la meilleure capacité à inhiber la croissance des 2 souches d'*Aspergillus* ainsi que la meilleure capacité détoxifiante (diminution de 74% d'AFB_1 et 92.5% d'OTA). Une autre souche de *Bacillus*, MHS 13, capable à la fois d'inhiber la croissance des 2 souches d'*Aspergillus* et de diminuer de 85% la teneur en AFB_1 a été identifiée comme *Bacillus subtilis*.

Finalement, le surnageant de culture et l'extrait cellulaire des 2 souches de *Bacillus* ont été testés pour la dégradation de l'AFB_1 et de l'OTA afin de mieux comprendre les mécanismes de la détoxification. Les conditions optimales de la dégradation ont aussi été étudiées. Les résultats montrent que l'OTA était significativement degradée par le surnageant de *Bacillus licheniformis* CM 21 ($p<0.0001$) en OTalpha. Le pourcentage de dégradation d'OTA a été de 97.5% et les conditions optimales d'activités étaient : pH 7.0, 37°C, 24 h de croissance de la bactérie et 2 heures d'incubation avec la solution d'OTA. De même, l'OTA a été

degradée à 69% par le surnageant de *Bacillus subtilis* MHS 13 ($p<0.0017$) à pH 5.0, 37°C, 48 h de croissance de la bactérie et 2 heures d'incubation avec la solution d'OTA. Le mécanisme de dégradation proposé est donc la présence d'une carboxypeptidase A extracellulaire. En effet, le contenu intracellulaire des 2 bactéries n'a montré aucune activité détoxifiante.

Par ailleurs l'AFB_1 n'a été dégradée ni par le surnageant de culture, ni par le contenu extracellulaire des 2 bactéries. On peut donc supposer que le mécanisme de détoxification pour l'AFB_1 n'est pas de nature enzymatique.

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Awanwee Petchkongkaew

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LIST OF SYMBOLS AND ABBREVIATIONS

AFLs	=	Aflatoxins
AFB_1	**=**	Aflatoxin B_1
AFM_1	=	Aflatoxin M_1
OTA	**=**	Ochratoxin A
OTB	=	Ochratoxin B
OTC	=	Ochratoxin C
OTα	**=**	Ochratoxin α
°C	=	Degree Celsius
h	=	Hour
min	=	Minute
sec	=	Second
g	=	Gram
kg	=	Kilogram [10^3 gram]
mg	=	Milligram [10^{-3} gram]
µg	=	Microgram [10^{-6} gram]
ng	=	Nanogram [10^{-9} gram]
v	=	Volume
M	=	Molar [$Molel^{-1}$]
µmol	=	Micromole [$Mole^{-6}$]
N	=	Normality

LIST OF SYMBOLS AND ABBREVIATIONS (Continued)

OD	=	Optical density
ppm	=	Part per million
ppb	=	Part per billion
rpm	=	Revolution per minute
LD_{50}	=	Median lethal dose
i.p.	=	Intraperitoneal
w	=	Weight
ml	=	Milliliter [10^{-3} liter]
µl	=	Microliter [10^{-6} liter]
psi	=	Pound per square inch
Phe	=	Phenylalanine
DNA	=	Deoxyribonucleic acid
RNA	=	Ribonucleic acid
t-RNA	=	Transfer-Ribonucleic acid
m-RNA	=	Messenger-Ribonucleic acid
IgG	=	Immunoglobulin G
IgA	=	Immunoglobulin A
IgM	=	Immunoglobulin M
HPLC	=	High Performance Liquid Chromatography
mm	=	Millimeter [10-3 Meter]
nm	=	Nanometer [10^{-9} Meter]

CHAPTER I

INTRODUCTION

The soybean is one of the most economical and valuable agricultural commodities because of its unique chemical composition, high nutritional value and low cost. Among cereal and other legume species, it has the highest protein content (around 40%) (Liu K., 1997). The soybean also contains about 20% oil. Other valuable components found in soybeans include water, phospholipids, vitamins, minerals and minor substances, some of which, such as trypsin inhibitors, phytase and oligosaccharides, are known to be biologically active. Others, such as isoflavones, are just being recognized for their powerful ability to prevent human cancers, osteoporosis, heart disease, kidney disease, lower cholesterol and inhibit bone resorption. Additionally, the nutritional value of the soybean is the inhibitory activity of an angiotensin 1-converting enzyme (ACE) detected in soybean has been found to play an important role in regulating blood pressure and salt and water balance.

Substituting soyfoods, for example Japanese natto, Thai Thau-Nao, Kinema, Dawadawa, for many animal products will therefore result in some nutritional advantages because they are an excellent source of high quality protein, are low in saturated fat, and are cholesterol-free. Most of people in Japan always consume natto because it is a highly nutritious food. Through fermentation, protein of cooked soybeans is degraded into amino acid, thus improving digestibility. In addition, the

riboflavin and vitamin B_{12} content of natto is three and five times greater, respectively, than in cooked soybeans.

Thua-Nao is one of the oldest traditionally fermented soybean products produced for years by the people in many small villages up north of Thailand. *Bacillus* spp., a Gram-positive, strict or facultative aerobe and endospore-forming bacteria, was found to be the dominant microflora of this product (Chantawannakul et al., 2002).

Unfortunately during the production of fermented soybean, the raw materials (soybeans) would be sometimes contaminated with *Aspergillus* spp. which the intrinsic and extrinsic factors may also induced the mycotoxin formation, lead to the health risk of consumers. If the consumers take moderate to low mycotoxin concentrations, in the long term much more serious problems may arise

This research aimed to monitor some factors that will inhibit mycotoxin formation in order to control the mycotoxin contamination level during soybean fermentation. For that, we focused on the interaction between *Bacillus* spp., which is used as starter culture of soybean fermentation product, and the contaminant, *Aspergillus* spp on one hand. On the other hand, we will study the effect of *Bacillus* on mycotoxins.

Among food, another important mycotoxin is ochratoxin A (OTA) which contaminates many products such as cereals, grapes, cacao, coffee, etc. Up to now no efficient method has been found to remove OTA from contaminated food and it would be of great interest to find enzymes which take part in a detoxifying process.

1.1 Research objectives

1. To study the interaction between *Bacillus* spp. and *Aspergillus flavus*: inhibition of growth and aflatoxin production/detoxification.

2. To study the effect of *Bacillus* spp. on *Aspergillus westerdijkiae* NRRL 3174 and ochratoxin A detoxification.

1.2 Research hypothesis

1. *Bacillus spp.* which used as a starter culture in soybean fermentation product can inhibit the growth of *Aspergillus flavus* and/or aflatoxin B_1 production.

2. *Bacillus spp.* which used as a starter culture in soybean fermentation product can inhibit the growth of *Aspergillus westerdijkiae* NRRL 3174.

3. *Bacillus* spp. is able to remove both aflatoxin B_1 and ochratoxin A from contaminated food.

1.3 Scope and limitation of the study

This research will attend on soybean fermentation products especially fresh Thua-Nao.

1.4 Expected results

1. To observe the optimum condition that results in the mycotoxins reduction during soybean fermentation and will provide safe Thua-Nao and the quantity control of mycotoxins in the acceptable level and so having no adverse effect to consumers.

2. To gain knowledge of the interaction between *Bacillus* spp., *Aspergillus flavus* and *Aspergillus westerdijkiae* NRRL 3174 and the action of *Bacillus* spp. on mycotoxins.

1.5 References

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CHAPTER II

LITERATURE REVIEW

2.1 Soybean

The soybean (*Glycine max*), a native of China, is one of the oldest crops of the Far East. For centuries, the Chinese and other Oriental people, including Japanese, Korean, and Southeast Asians, have used the bean in various forms as one of the most important sources of dietary protein and oil. Many simple and yet effective cooking processes have been developed to make a variety of wholesome and nutritious soybean dishes. There are fresh, dried, toasted, powdered, liquid and moist, and paste products available, and they are used in meals throughout the year as cooking materials and seasonings.

2.1.1 Nutritional significance and health beneficial factors in soybeans

Soybeans are also rich in other nutrients, such as essential fatty acids, calcium, iron, zinc, and fat-soluble vitamins. The nutritional contents of soybean can be altered significantly according to how soy foods are processed from soybean. Since Tempeh and Natto are made from whole beans, the nutrient contents are closer to that of whole beans. Sometimes processing actually improves the nutritional value of soy foods. For example, during fermentation of soy foods, degradation of protein takes place, producing amino acids, and thus the digestibility of protein increases. Table 2.1 shows that soy foods make a significant contribution to nutrient needs.

Table 2.1 Nutrient contents of soy foods (per 100 grams).

Soy foods	Protein (g)	Lipid (g)	Carbohydrate (g)	Fiber (g)	Ca (mg)	Fe (mg)	Zn (mg)
Soybean[a]	41.3	17.6	21.6	4.7	250	7.6	4.84
Soy sauce [a]	4.3	0.4	4.4	0.0	62	5.2	N/A
Ko chu jang [a]	8.9	4.1	15.9	3.5	126	13.6	N/A
Soy paste [a]	12.0	4.1	10.7	3.8	122	5.1	3.12
Natto [b]	17.7	11.0	12.8	1.6	217	8.6	3.03
Chongkuk jang [a]	16.5	10.0	9.8	2.3	90	3.3	N/A
Tempeh [b]	19.0	7.7	14.0	3.0	95	2.3	1.81
Soy curd [a]	8.6	5.5	1.7	0.3	181	2.2	0.88
Soybean sprout [a]	3.4	0.9	2.3	0.5	32	0.8	N/A

N/A – data not listed or unavailable

[a] source: Korea Rural Nutrition Institute. Food composition Table (1991)

[b] source: USDA. Composition of foods (1986)

Soy foods are also high in fiber. Soybean hulls are reported to contain about 87% dietary fibers, consisting of 40-53% crude cellulose, 14-33% hemicellulose, and 1-3% lignin on a dry basis. Therefore, whole soybean foods such as Tempeh, are a good way to get fiber. However, soy curd and other products made from soymilk are very low in fiber, since that part of soybean is eliminated in the processing.

Soy foods give us many ways to add the health benefits of soy to our diets. That is, consuming soy foods as part of the normal diet would be a wise nutritional practice because soybean provides several nutrients and helps prevent cancer, osteoporosis and cardiovascular diseases.

Explanations of how soy protein lowers blood cholesterol include: 1) a decrease in cholesterol absorption and increase in bile acid excretion 2) an increase in liver low density lipoprotein (LDL) receptors and faster clearance of LDL from the blood 3) a decrease in hepatic cholesterol synthesis 4) an increase in blood thyroxin and thyroid stimulating levels. The relative mixture of amino acids and a lower ratio of lysine to arginine in soy protein are considered to be responsible for part, but not all, of the physiological changes with soy intake.

Fibers may be another one. Soluble dietary fibers are effective in lowering plasma triglyceride and cholesterol concentrations. The metabolic effect of soybean fiber for animals was tested by several researchers. The overall results suggest beneficial effects on lipid metabolism with moderate intakes of soybean hulls as a source of dietary fiber. Genistein may partly contribute to fewer occurrences of heart diseases, since it is known to inhibit the activity of thrombin, an enzyme that is responsible for causing blood components called platelets to form clots. Various other compounds in soy, such as lecithin, saponins, and phytosterols, may also contribute to soy's hypocholesterolemic effects.

Soy foods are rich in anticarcinogens, substances that in some way prevent or control cancer. Protease inhibitors prevent the activation of the specific genes that cause cancer. They also protect against the damaging effects of radiations and free radicals, which can attack DNA. Phytate is the storage form of the mineral

phosphorus, found in foods that are in fiber. Phytate may help to prevent cancer by enhancing the immune system, in particular by increasing the activity of natural killer cells, which can attack and destroy cancer cells and tumors. Saponins are antioxidant and so protect us from the damaging effects of free radicals. Saponins also prevent mutations that can lead to cancer. Phytosterols may protect colon against the harmful effects of bile acids, reducing the development of colon tumors by as much as 50%. Phytosterol intake is inversely related to occurrence of colon cancer.

Soybeans are extremely rich in a unique group of phytoestrogens called isoflavones. The isoflavones in soybean also explain why soy foods may lower the risk of cancer. Isoflavones work in ways that make them effective against a wide range of cancers. The major isoflavones in soybeans are genistein and daidzein. The main isoflavone in soybeans, genistein, has an attribute that makes it a potent weapon in the fight against cancer. Genistein is effective against so many different types of cancers because the mechanism by which it appears to operate affects very fundamental aspects of the cancer process. Genistein not only inhibits cancer cell growth but also causes cancer cells to differentiate, that is, to go from a cancer to a normal cell.

Osteoporosis is a significant problem in elderly woman. Osteoporosis results because bone resorption proceeds faster than bone formation and there is a net loss of bone. Ipriflavone, a derivative of naturally occurring isoflavones, is a drug which inhibits bone resorption and enhances bone formation, resulting in an increase in bone density. Furthermore, genistein has estrogenic activity. Thus soy helps to prevent osteoporosis by two ways-by daidzein affecting bone resorption and formation directly and by genistein acting as a weak estrogen.

Soybeans are also free of milk sugar, that is, lactose. Therefore soy foods provide a wonderful selection of alternatives to dairy foods for people who are lactose intolerant. Moreover, soybean sugars are so effective in promoting the growth of bifido-bacteria which play a very important role in promoting health of the colon when these oligosaccharides are replacing common table sugar. Finally, it has been known for centuries among Koreans that the soybean sprout soup has the anti-hangover function also.

2.2 Fermentation and alkaline fermentation

The word “Fermentation” is derived from the Latin meaning “to boil” since the bubbling and foaming of early fermenting beverages seemed closely akin to boiling. Fermentation is the chemical transformation of organic substances into simpler compounds by the action of enzymes, complex organic catalysts, which are produced by microorganisms such as mold, yeasts, or bacteria. Enzymes act by hydrolysis, a process of breaking down or predigesting complex organic molecules to form smaller (and in the case of foods, more easily digestible) compounds and nutrients.

Most fermentation is activated by either molds, or yeasts, or bacteria, working singularly or together. The great majority of these microorganisms come from a relatively small number of genera; roughly eight genera of molds, five of yeasts, and six of bacteria. An even smaller number are used to make fermented soyfoods: the molds are *Aspergillus*, *Rhizopus*, *Mucor*, *Actinomucor*, and *Neurospora* species; the yeasts are *Saccharomyces* species; and the bacteria are *Bacillus* and *Pediococcus*

species plus any or all of the species used to make fermented milk products (Steinkraus, 1983).

Fermented foods are food substrates that are invaded or overgrown by edible microorganisms whose enzymes, particularly amylases, proteases, lipases hydrolyze the polysaccharides, proteins and lipids to nontoxic products with flavors, aromas and textures pleasant and attractive to the human consumer. If the products of enzyme activities have pleasant odors or undesirable, unattractive flavors or the products are toxic or disease producing, the foods are described as spoiled.

Fermentation plays at least five roles in food processing:

1) Enrichment of the human dietary through development of a wide diversity of flavors, aromas and textures in food.

2) Preservation of substantial amounts of food through lactic acid, alcoholic, acetic acid, alkaline fermentations and high salt fermentations.

3) Enrichment of food substrates biologically with vitamins, protein, essential amino acids and essential fatty acids.

4) Detoxification during food fermentation processing

5) A decrease in cooking times and fuel requirements.

2.2.1 Classification of food fermentations

Steinkraus (1997) classified fermentations according to the following categories;

1. Fermentation producing textured vegetable protein meat substitutes in legume/cereal mixtures

2. High salt/ savory meat-flavored/ amino acid/ peptide sauce and paste fermentations
3. Lactic acid fermentations
4. Alcoholic fermentations
5. Acetic acid/ vinegar fermentations
6. Alkaline fermentations
7. Leavened breads
8. Flat unleavened breads

Steinkraus has found the above classification useful and a way of predicting what microorganisms may be involved and what chemical, physical and nutritive changes may occur in new unfamiliar fermented foods. The classification also related well to safety factors found in fermented foods.

2.2.2 Alkaline fermentations

Fermentations involving highly alkaline fermentations are generally safe. Alkaline fermentations involving *bacilli* fermenting protein rich beans and seeds are of considerable importance in widely separated parts of the world. Africa has a number of very important foods/ condiments that are not only used to flavor soups and stews but also serve as low-cost sources of protein in the diet (Odunfa, 1989). Among these are Nigerian dawadawa, Ivory cost soumbara and West African iru made by fermentation of soaked, cooked locust bean *Parkia biglobosa* seeds with bacteria belonging to genus *Bacillus*, typically *Bacillus subtilis*. Nigerian ogiri made by fermentation of melon seed (*Citrullus vulgaris*); Nigerian ugba made by fermentation of the oil bean (*Pentacletha macrophylla*); Sierra Leone ogiri-saro made

by fermentation of sesame seed (*Sesamum indicum*); Nigerian ogiri-igbo made by fermentation of castor bean (*Ricinus communis*) seeds and Nigerian ogir-nwan made by fermentation of the fluted pumpkin bean (*Telfaria occidentale*) seeds. Soybeans can be substituted for locust beans.

This group also includes Japanese natto, Thai Thua-Nao and Indian kenima all based upon soybean.

The essential microorganisms are *Bacillus subtilis* and related *bacilli*. The organisms are very proteolytic and the proteins are hydrolyzed to peptides and amino acids. Ammonia is releases and the pH rapidly reaches as high as 8.0 or higher. The combination of high pH and free ammonia along with very rapid growth of the essential microorganisms at relatively high temperatures above 40°C make it very difficult for other microorganisms that might spoil the product to grow. Thus, the products are quite stable and well-preserved especially when dried. They are safe foods even though they may be manufactured in an unhygienic environment.

2.3 Thua-Nao

Thua-Nao, a traditionally non-salted fermented soybean, is locally consumed by people in the northern of Thailand and utilized as a substitute for fish paste. The traditional production of Thua-Nao involves predominantly *Bacillus spp*. fermentation on soybean substrates. It is a high protein fermented with ammonia smell but without vitamin B_{12} content. Yongsmith (1999) found that used of mixed culture fermentation of oligosaccharides, utilizing *Bacillus subtilis* and vitamin B_{12} producing *Bacillus megaterium*, has improved the quality of Thua-Nao. Soybean oligosaccharide sugar utilizing *Bacillus* was isolated from various sources of Thua-Nao. *Bacillus subtilis* B_4

was found to produce the best proteolytic activity, as well as soybean sugar-utilizing property, among 120 *Bacillus* isolates. Mixed fermentation of *Bacillus* B_4 and *Bacillus megaterium* under optimum conditions, could improve the quality of Thua-Nao by enhancing more digestible soybean proteins, soybean sugar, as well as vitamin B_{12} content in the fermented masses.

To produce traditional Thua-Nao, soybeans are washed and soaked in water overnight. Soaked beans are then cooked by boiling for 3-4 h, gently grounded, placed in the basket lined with banana leaves and left to ferment naturally for 2-3 days at ambient temperature. After the fermentation, fresh Thua-Nao can be consumed directly by steaming. Besides, it is also popular-due to a long shelf life- to store in a dried form by exposing to sunlight and these dried products are a major condiment in various kinds of local dishes.

Thua-Nao is considered as an inexpensive high-protein food that could be easily produced, and if properly prepared has a long shelf life. Thua-Nao paste and chips contain 16.9 percent and 36.8 percent of protein and 7.4 percent and 14.8 percent fat, respectively.

2.4 Aflatoxin

Mycotoxins are produced mainly by the mycelial structure of filamentous fungi, or more specifically, the molds. Mycotoxins are secondary metabolites that have no biochemical significance in fungal growth and development (Moss, 1991). Toxigenic molds are known to produce one or more of these toxic secondary metabolites. It is well established that not all molds are toxigenic and not all secondary metabolites from molds are toxic.

According to current estimates, about 100,000 molds have been identified, from which over 400 can be considered potentially toxic and of which about 5% are known to produce toxic compounds or classes of compounds causing problems in one or more parts of the world. Toxic metabolites were found to occur naturally on foods and feeds from cereals (corn, barley, grain sorghum, oats, rice, triticale, rye, wheat), pulses, legumes, soybean, peanut, etc.

Feeds contaminated with fungal toxins (mycotoxins) pose a health risk to animals and, as a consequence, may cause big economical losses due to the lower efficacy of animal husbandry. In addition, directly or indirectly (carry through to animal products), contaminated foods may also pose a health risk to humans. Examples of mycotoxins of greatest public health and agronomic significance include aflatoxins, ochratoxins, trichothecenes, zearalenone, fumonisins, tremorgenic toxins, and ergot alkaloids.

Aflatoxins are a group of chemically related mycotoxins, which are produced by particular species of molds. Their name derives from the fungus *Aspergillus flavus* on which much of the early work with these substances was performed (i.e. the genus *Aspergillus*, the species flavus and the suffix toxin).

Subsequent research revealed that aflatoxins are produced by strains of *A. flavus* and strain of the related species *A. parasiticus*, *A. nominus* and *A. niger*. Furthermore, it was discovered that there are a number of distinct, but structurally related aflatoxin compounds -the four most commonly seen being designated B_1, B_2, G_1, and G_2. The B designation of aflatoxins B_1 and B_2 resulted from the exhibition by these compounds of blue fluorescence under the ultraviolet (UV)-light, whereas the G designation refers to yellow-green fluorescence under UV-light. The various

subscripts refer to the corresponding chromatographic R_f values in silica gel and thin layer chromatographic systems, with chloroform-methanol (97:3) as the developer. In the case of aflatoxins M, the R_f values are based on formamide-impregnated Whatman No.1 filter paper, with ethyl acetate-benzene (9:1) as the developer (Asao, 1965).

Aflatoxins M_1 and M_2, are hydroxylated derivatives of aflatoxin B_1 and B_2 that may be found in milk, milk products, or meat (hence the designation M). They are formed by metabolism of B_1 and B_2 in the body of the animals, following absorption of contaminated feed. Aflatoxin B_1 is the most frequent of these compounds present in contaminated food samples and aflatoxins B_2, G_1 and G_2 are generally not reported in the absence of aflatoxin B_1. Figure 2.1 represents the structure of aflatoxin B_1 and related aflatoxins.

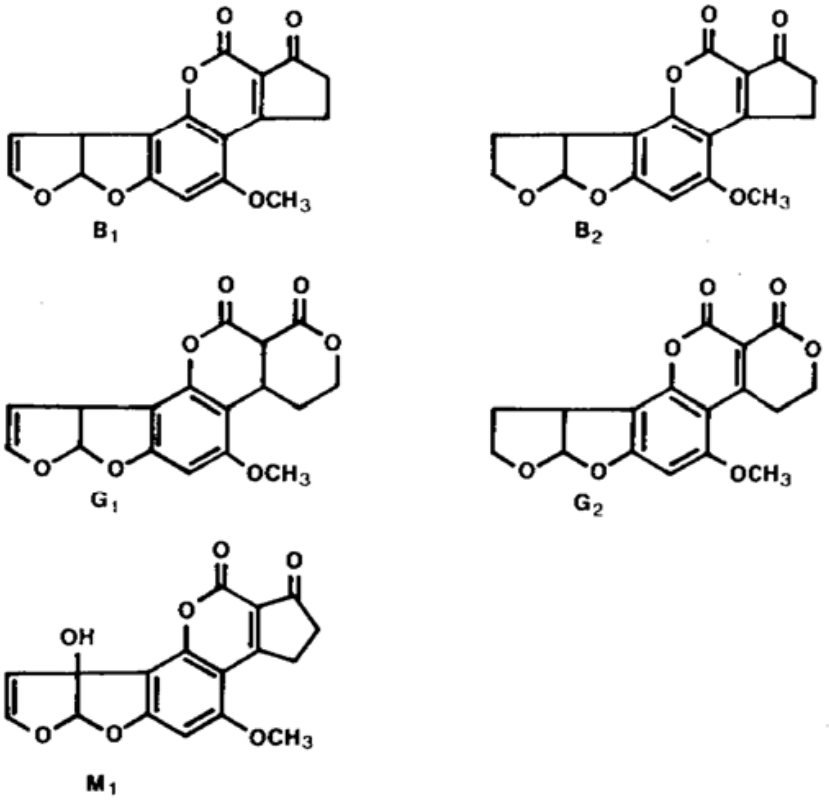

Fig. 2.1 Structure of aflatoxin B_1 and related aflatoxins.

2.4.1 Occurrence

Thailand sent Costa Rica 200,000 tones of maize, which were contaminated with aflatoxins. Part of this was destroyed but some was sent to Nicaragua and Venezuela. In the 1,100 tones sent to Nicaragua, aflatoxin contamination between 150 and 300 μg/kg was detected and the shipment was destroyed. Venezuela rejected the consignment, adding the cost of transport and handling back to port of origin to the losses mentioned above.

Peanut cakes exported by Brazil to Europe were contaminated with up to 2,000 to 3,000 μg/kg of aflatoxin B_1, prohibiting their use for cattle fodder. Some of these consignments were used as fertilizers. Discounts were in some cases 75%. In 1975, trading of these goods between Brazil (exporter) and France, Belgium and The Netherlands (importers) was suspended (Feuell, 1966).

In 1981, 95% of the bulk peanuts imported by the USA from Brazil showed an aflatoxin contamination higher than 25 μg/kg (Bhat, 1977). In the environmental monitoring report of the UNEP/FAO/WHO Program, it was stated that the other peanut exporting country that year was Argentina, with 5% contaminated samples at a level above 25 μg/kg

In Barbados, in 1986, 10% of the peanut harvest was burned in view of levels of aflatoxins above 450 μg/kg. The rapid deterioration of the peanuts, which brings about great economic losses, decreased the planted area from 350 acres that year to 30 acres in 1990. At present, production does not cover demand and peanuts are imported from Brazil

In India, there are reports of aflatoxin B_1 levels as high as 26,700 μg/kg for groundnut cake and 520 μg/kg for cottonseed cake (Strange, 1991).

In certain countries in the tropics, analyses have shown significant aflatoxin contamination of foodstuffs consumed. For example, Campbell (1970) found that locally produced peanut butter in the Philippines contained significant amounts of aflatoxin (median level, 500 μg/kg), and aflatoxin M_1 was found in the urine of children consuming 60 g or more of contaminated peanut butter.

Shank et al. (1972) found that many food products in Thailand, which appeared wholesome and fit for human consumption, contained relatively high levels of aflatoxin. For example, the aflatoxin content of dried fish, dried chili peppers, corn, and peanuts was shown to be 772, 996, 2700, and over 12,000 ppb, respectively. In some regions, as much as 16% of the samples of foodstuffs were contaminated with aflatoxins. In three areas, the calculated average aflatoxin intakes on a family basis were 73 to 81 ng, 45 to 77 ng, and 5 to 8 ng/kg body weight/day.

In one study, 38% of samples of sorghum in Uganda were found to be positive for aflatoxins, with total levels ranging from 1 to > 1,000 μg/kg.

In many parts of the world, particularly in the tropics, many cases of human poisonings strongly implicate aflatoxin consumption. In Taiwan, 26 persons were poisoned following consumption of moldy rice for up to 3 weeks. Two samples of moldy rice contained approximately 200 μg/kg aflatoxins. Three poisoned children died. The victims suffered edema of the legs, abdominal pain, vomiting, and palpable liver, but no fever (Ling, 1967).

A case in Uganda involved a 15-year-old boy who died of symptoms resembling the case in Taiwan following consumption of diet which included moldy cassava. The cassava sample contained 1.7 mg aflatoxin/kg. Two other siblings were likewise affected but recovered (Serck-Hanssen, 1970). The autopsy findings revealed

liver necrosis and mild fatty liver, in addition to other pathological changes. Based on monkey studies, a lethal amount of aflatoxin in the cassava might have been consumed if the victim ate the moldy cassava for a longer period (Alpert et al., 1970).

A case of Reye's syndrome reported from Thailand may have involved aflatoxin poisoning (Bourgeois, 1971). This case involved a three-year-old boy who died of Reye's syndrome following consumption of moldy rice containing as much as 10 mg total aflatoxins/kg. The similarity between Reye's syndrome and acute aflatoxicosis in the macaque monkey is striking (Bourgeois, 1971). In the monkey suffering from aflatoxicosis, aflatoxin B_1 was demonstratably present in tissue up to 6 days after administration of the mycotoxin (Shank, 1971). In Reye's syndrome, one or more autopsy specimens from victims also contained aflatoxin B_1, and in two cases, the level of aflatoxin was comparable to that in monkeys given and LD_{50}-dose of the mycotoxin (Shank, 1971). Two additional cases of Reye's syndrome in which aflatoxin were found in the tissue were reported from New Zealand. (Becroft and Webster, 1972). Table 2.2 and 2.3 show the occurrence of *Aspergillus* spp. and aflatoxin in some agricultural commodities. Moreover, table 2.4 shows the occurrence of aflatoxin in peanuts and peanut products.

Table 2.2 Occurrence of *Aspergillus* in some agricultural commodities (Rustom, 1997).

Commodity	Country	Species
Peanut	Sudan	*A. flavus*
	Egypt	*A. flavus*+*A. niger*
	South Africa	*A. flavus*+*A. parasiticus*
Maize	India	*A. flavus*
	China	*A. flavus*
	Uganda	*A. flavus*+*A. parasiticus*
	Nigeria	*A. flavus*+*A. parasiticus*+*A. niger*
	USA	*A. flavus*
Wheat	China	*A. flavus*
	Russia	*A. flavus*
Rice	China	*A. flavus*
	India	*A. flavus*+*A. parasiticus*
Millet	India	*A. flavus*+*A. parasiticus*
Soybean	Argentina	*A. flavus*+*A. parasiticus*
Sunflower oil	China	*A. flavus*
	Russia	*A. flavus*
Coconut	India	*A. flavus*
Pistachio nuts	USA	*A. niger*+*A. flavus*+*A. parasiticus*
	Turkey	*A. flavus*
Figs	Switzerland	*A. flavus*+*A. parasiticus*

Table 2.3 Occurrence of aflatoxins in some food and feeds (Rustom, 1997).

Food	Country	Contaminated/ Total examined	Aflatoxin	Concentration (µg/kg)
Rice	China	33/252	B_1	5-50
	India	1/1	B_1	20
Maize	Kenya	70/78	-[a]	30-920
	USA	2370/2633	-	10-700
	Mexico	86/96	-	2.5-30
	Brazil	40/328	B_1	>20
	Denmark	6/197	Total[b]	5-174
	France	3/3	B_1	20
	India	47%	B_1	>20
Sorghum	South Africa	-	B_1	0-25
	India	-	B_1	7-75
Copra	Tonga island	1/2	B_1	37
Millet	India	49/75	B_1	17-2110
Soybeans	USA	11/11	B_1	<20
		3/24	B_1	<6
	Argentina	9/94	B_1	1-36

[a] -, not mentioned.

[b] $B_1+G_1+B_2+G_2$.

Table 2.3 Continued.

Food	Country	Contaminated/ Total examined	Aflatoxin	Concentration (µg/kg)
Oat, barley and wheat	Sweden	20/116	B_1	50-400
Meat	Egypt	9/150	B_1,B_2	4-15, 2-25
Dried figs	UK	8/93	Total	10-40
	Sweden	16/27	-	5-67
Potato	Fiji island	7/20	B_1	6-12
Linseed	India	46/105	B_1	120-810
Bakery products	Spain	1/50	B_1,G_1	67,46
Cottonseed	Argentina	5/5	B_1	20-200
Sunflower	Spain	1/1	B_1	20
Mustard seed	India	44/100	-	75
Milk	Sweden	19/267	M_1	>0.05
	Spain	14/47	M_1	20-100
Human milk	Abu Dhabi	443/445	M_1	0.002-3.0

[a]-, not mentioned.

[b] B_1+G_1+B_2+G_2.

Table 2.4 Occurrence of aflatoxin in peanuts and peanut products (Rustom, 1997).

Food	Country	Contaminated/ Total examined	Aflatoxin	Concentration (µg/kg)
Peanuts	Argentina	3/3	B_1	20-200
	Senegal	72/72	B_1	20-200
	Brazil	940/1044	-[a]	30-5000
		20/20	B_1	80-6450
		26/86	B_1,G_1	10-2000, 20-800
	Mexico	26/29	-	700
	USA	108/120	-	24
	Philippines	56/98	-	3-2888
	Fiji island	11/22	B_1	1-18
	India	10/20	B_1	33-440
Butter	China	1/2	B_1	20
	UK	69/77	-	38-535
	USA	246/2510	Total[b]	20-100
Cake	Nigeria	28/32	B_1	37-455
	USA	223/401	Total	31-100
Snacks	India	31%	B_1	2-1500

[a] -, not mentioned.

[b] $B_1+G_1+B_2+G_2$.

2.4.2 Mode of toxicity

The adverse biological properties of aflatoxin seen in poisoning episodes in animals can be categorized in two general forms.

2.4.2.1 Acute aflatoxicosis

Acute aflatoxicosis occurs following the ingestion of high doses of aflatoxins over a relatively short period of time. Specific acute episodes of disease may include haemorrhage, acute liver damage, oedema, alteration in digestion, absorption and/or metabolism of food, and possibly death. The classical assessment of toxicity of compounds centers on the determination of LD_{50} values. These values of acute toxicity are subject to wide variation depending, for example, on factors such as age, sex, and size of animals. Flannigan (1991) lists LD_{50} values for AFB_1 ranging from 1.0 to 17.9 mg/kg body weight in laboratory animals, but 1-day-old duckling are particularly sensitive, with a value of 0.5 mg/kg. In adult ruminants, exposure to aflatoxins can depress feed efficiency, immunocompetence and reproductive performance, as shown by studies with dairy cattle (Diekman and Green, 1992). The effects on feed efficiency presumably arise from impaired ruminal function, including reduced cellulose digestion, volatile fatty acid production and motility. In dairy cattle another problem arises from the transformation of AFB_1 to a related metabolite, aflatoxin M_1 (AFM_1) which is secreted in the milk. The conversion at high and low levels of AFB_1 contamination of dairy feeds hasbeen reviewed by van Egmond (1989) who concluded that AFM_1 is both hepatotoxic and carcinogenic. In quantitative terms, its toxicity for ducklings and rats appears to be similar to that of AFB_1, but its

carcinogenic potential is estimated to be less than that for AFB_1. Table 2.5 shows toxic effects of aflatoxin in various animal species.

2.4.2.2 Chronic aflatoxicosis

Chronic aflatoxicosis occurs following the ingestion of low to moderate doses of aflatoxins over a prolonged period. The effects may be subclinical or difficult to recognize. Some of the more frequently described symptoms include impaired food conversion and slower rates of growth, with or without the occurrence of an overt aflatoxin syndrome as seen with acute poisoning. Underlying these symptoms is a chronic poisoning of the liver leading ultimately in cirrhosis and/or liver cancer.

2.4.2.3 The effect of aflatoxins in human health

In general, effects of mycotoxins on humans are limited to case studies. Similar to rat studies and *Salmonella* assays for mutagenicity, case studies on AF exposures have provided insight to toxicity in humans. Acute AF exposures have been associated with epidemics of acute toxic hepatitis in area of China and Africa with death rates ranging from 10 to 60% (Bhat and Krishnamachari, 1977). Studies on an individual attemping suicide by ingesting purified AFLs have demonstrated that single doses are not as effective in humans as long-term doses (Willis et al., 1980). Toxicity symptoms in a young woman, who attempted suicide with AFLs in the amounts of 5.5 mg over 2 days and 35 mg over 2 weeks (6 months from the initial dose), included transient nonpruritic macular rash, nausea, and headache. The woman recovered completely and had no significant signs of liver injuries when examined 14 years later (Willis et al., 1980). Results of this case and others suggested that extended

subacute doses, as seen in dietary exposures in certain countries, might be required for inducing the lethal acute toxic effects (Willis et al., 1980).

The largest risk of AFLs to humans is usually the result of chronic dietary exposure. Such dietary AFLs exposures have been associated with human hepatocellular carcinomas, which may be compounded by hepatitis B virus. Approximately 250,000 deaths are caused by hepatocellular carcinomas in China and Sub-Saharan Africa annually (Groopman et al., 1992) and are attributed to risk factors such as high daily intake (1.4 μg) of AFLs (Wild et al., 1992) and high incidence of hepatitis B (Kensler et al., 1991). Aflatoxins have been found in tissues of children suffering from Kwashiorkor and Reye's syndrome and were thought to be a contributing factor to these diseases (Becroft and Webster, 1972). Reye's syndrome, which is characterized by encephalopathy and visceral deterioration, results in liver and kidney enlargement and cerebral edema (Becroft and Webster, 1972).

Table 2.5 Toxic effects of aflatoxin in various animal species (Eaton and Groopman, 1994).

Animals	Toxic Effects	
	LD_{50}	Histopathology
Poultry		
Duckling	B_1: 12 μg 18.2 μg M_1: 16.0 μg G_1: 39.2 μg M_2: 61.4 μg B_2: 172.5 G_2: 0.4 mg/kg	Extensive bilary proliferation in the liver and fatty degeneration of the peripheral parenchyma cells following intubation with 15 μg aflatoxin; proliferation of the bile duct epithelial cells, vacuolation of the focal parenchyma cells, slight to moderate fibrosis; liver infraction with 10-40 μg aflatoxin/day; mitochondria necrobiosis and regressive changes, reticular fiber proliferation; aflatoxin M_1 at high doses-hemorrhagic necrosis; at low doses-bile duct proliferation and extensive changes in the liver cells and renal tubular necrosis.
Ducks		Decreased liver weights with sub-lethal doses of aflatoxin B_1; renal parenchymal hemorrhage ; liver atrophy, liver tumors with doses of aflatoxin B_1; liver atrophy, liver tumors developed after 14 months feeding; lowest tumorigenic dose 0.03 ppm
Coturnix quails		Bile duct proliferation, slight to moderate fibrosis, hepatic cell vacuolation

Table 2.5 Continued.

Animals	Toxic Effects	
	LD_{50}	Histopathology
Chicken		Hydropic and fatty liver cells, cell vacuolation, slightly increased cell size, mild proliferation of ductules, hemorrhages in the liver, muscle necrosis with diffused increase of sarcolemnal nuclei; in Rhode Island Red
		Chicks, decrease in RNA and vitamin A and fats with 10 ppm aflatoxin B_1 for 3 weeks; decreased hatchability of eggs
Turkey poults		Liver, kidney, and myocardial congestion, duodenal catarrhal enteritis, retrogressive and regenerative changes in the liver parenchyma, swollen liver cells, vacuolation in some cells, necrosis in the perisinusoidal region, karyorrhexis, and karyolysis; grayish white surface and internal nodules throughout the liver with fibrosis and bile duct proliferation
Fish		
Rainbow trout		Hyperplasia of the bile duct epithelium and cholangitis; bile duct proliferation; hepatoma

Table 2.5 Continued.

Animals	Toxic Effects	
	LD_{50}	Histopathology
Rodents		
Rats	Oral, 7 mg/kg Oral, 5 mg/kg	Liver enlargement, brownish yellow irregular nodular surfaces, red and greenish cysts, yellowish focal lesions, macroscopic grayish lesions with zones of hemorrhages in the lungs; periportal liver necrosis ; hyperplastic foci and preneoplasticlesions ; atrophy of testicles, aspermatogenesis, retardation of fetal growth; tetarogenesis; hepatocarcinoma ; kidney tumors ; carcinoma of the glandular stomach Adenocarcinoma of the colon; malignant sarcomas and fibrocarcomas at the site of injection
Mice		Resistant to acute toxic effects even with high levels, but develop hepatomas; subcutaneous sarcomas; adenomatous pulmonary tumors
Guinea pigs	B_1, i.p.: 1.4 mg/kg	Centrilobular necrosis, biliary proliferation within 48 hr; lesions in the kidneys, adrenals, pancreas, and GI tract; severe edema and liver necrosis, hepatoma; kidney tubular reflux

Table 2.5 Continued.

Animals	Toxic Effects	
	LD_{50}	Histopathology
Hamsters		Liver lesions; teratogenesis
Ferrets		Yellowish, hemorrhagic liver, fatty infiltration, centrilobular necrosis, cellular vacuolation in the liver, bile duct proliferation, bile duct and hepatic cell tumors
Rabbits		Lethal dose is 65 ppm aflatoxin/kg body weight fed for 2 consecutive days; 40 μg/day given to 5-month-old rabbits developed anorexia, retarded growth, weight loss, and death by the fifth week of administration
Caniners		
Dog		Hepatic lesions; hepatitislike disease
Ruminants		
Sheep		Relatively resistant; but may develop hepatic parenchymal cell neoplasia, nasal carcinoma and nasal chondroma; decreased fertility

Table 2.5 Continued.

Animals	Toxic Effects	
	LD_{50}	Histopathology
Ruminants		
Cattles		Severe tenesmus, liver fibrosis, ascites, visceraln edema, centrilobular necrosis, ductal cell hyperplasia, occlusion of the centrilobular veins; bile duct proliferation, chronic endophlebitis of the centrilobular and hepatic veins, karyomegaly of some parenchymal cells, liver cirrhosis; epithelial nephritis and ulceration of the abomasums; at levels of 0.7-1.0 μg aflatoxin/kg of feed-decreased weight gains observed; gross evidence of liver damage at 0.7 mg or over/kg of feed – enlarged liver cell nuclei
Primates		
Rhesus monkey		Fatty liver and cirrhosis; biliary fibrosis, severe fatty changes in the parenchymal cells, soft enlarged livers, enlarged yellow kidneys with fat accumulation; viral hepatitislike necrotic lesions; lethal to the Macaca irus monkey at 50 μg aflatoxin B_1/kg body weight

2.4.3 Aflatoxin problems in fermented soybean products

Due to the unique ability of fungi to produce many organic metabolites with diverse chemical structures, hundreds of mycotoxins have been discovered in recent years. The biological effects of mycotoxins are as varied as their chemical structures; they cause acute and chronic toxicity, in addition to carcinogenic, mutagenic, and teratogenic effects. Recent investigations have shown that many mycotoxins are immunosuppressors. It is apparent that, whereas the majority of mycotoxins are produced by toxic strains in the *Aspergillus* and *Penicillium* genera, some of the potent toxins are produced by *Fusarium* and other fungi as well. The LD_{50} range and type of toxic effects vary considerably. Mycotoxins may affect liver, kidney, and many other organs and tissues including cardiovascular, central nerve, respiratory, reproductive, and hemopoietic systems (Schiefer, 1986).

Cereal products (corn and wheat), peanuts, cottonseeds (aflatoxin), and mixed feed appeared to be the foodstuffs most commonly contaminated with mycotoxins. However, since mycotoxins could be produced both in the field as well as during storage, as long as conditions are favorable, practically no foods can avoid mold contamination and thus possible mycotoxins.

Because of its potent carcinogenic effect and wide distribution of *A. flavus* and *A. parasiticus* in the environment, aflatoxin B_1 (AFB_1) has been one of the most extensively studied mycotoxins. The aflatoxin problem continues to be a threat to human and animal health even after more than 30 years of investigation. The question of whether aflatoxins and/or other mycotoxins may be associated with traditionally fermented foods has been raised ever since the discovery of aflatoxins, particularly because *A. flavus* is related morphologically to the well known *A. oryzae*, which has

been used in the preparation of koji, a starter for a number of Oriental fermented foods. Extensive studies in several laboratories have revealed that the industrial koji inoculum, *A. oryzae*, does not produce aflatoxins (Hesseltine et al., 1966). Although it has been reported that a variant *A. oryzae* NRRL 1988 produced aflatoxins B_1, B_2, G_1, and G_2, on cowpeas or rice (El-Hag and Morse, 1976), the validity of such findings has been challenged by other investigators (Fennell, 1976). The inability of *A. oryzae* to produce AFB_1 was confirmed by Wei and Jong (1986) when they tested 169 strains of fungi within the *A. flavus* group in the ATCC reference collection for their ability to produce aflatoxins in three different media. Aflatoxins were produced in one or more of the media tested by 41% of *A. flavus*, 33% *A. flavus* var. *columnaris*, and 85% of *A. parasiticus* for a total of 59 positive strains of the 169 tested. None of the koji molds (i.e., *A. oryzae*, *A. sojae*, and *A. tamari*) produced detectable aflatoxin.

Whereas *A. oryzae* koji has been considered to be safe for use as a starter culture in the preparation of a number of Oriental fermented foods, *A. flavus* and other mycotoxin-producing fungi have occasionally been isolated from some of the fermented foods (Table 2.6). Occasionally aflatoxins were also detected in some of these indigenous fermented products (Alozie et al., 1979). These fungi may have originated in the raw materials carried through the fermentation or in contamination during fermentation and/or storage due to lack of sanitation. The existence of the toxicogenic fungi thus increases the risk of production of mycotoxins in fermented foods.

Raw materials such as rice, cereal products, and peanuts that have been used in the preparation of fermented foods are good substrates for the production of mycotoxin. Although studies on the production of aflatoxins in soybeans are

contradictory (Sherertz et al. 1976), production of aflatoxins in this commodity may vary with the varieties, methods of pretreatment, as well as the type of strain and the size of the inoculum (Shotwell and Hesseltine,1983).

Table 2.6 Occurrence of toxicogenic fungi in indigenous fermented foods.

Fermented foods	Fungi	References
Katsuobushi	*Aspergillus flavus*	Chu (1977)
	A. glacus group, *A. ochraceus*	Saito et al. (1974)
	A. ostianus	
	Penicillium cyclopium	
	P. putterillii	
Kaomak	*A. flavus, A. parasiticus*	Sripathomswat and
(fermented rice)		Thasnakorn (1981)
Taotjo (soybean sauce)	*A. terreus, A. fumigatus*	Sripathomswat and
	A. clavatus	Thasnakorn (1981)
Meju (or kokja)	*A. clavatus, A. flavus*	Kim (1971), Kim et al. (1977)
	A. ochraceus, A. versicolor	
Miromi (mash)	*A. flavus, P. uticae*	Saito et al. (1974),
		Udagawa et al. (1970)

Table 2.6 Continued.

Fermented foods	Fungi	References
Miso	*Alternaria* sp., *A. flavus*, *A. glaucus group*, *A. ostianus*, *A. sulphureus*, *A. terreus*, *A. versicolor*, *P. brevicompacum*, *P. charlesii*, *P. meleagrinum*, *Paecilomyces varioti*, *A. ochraceus*, *P. cyclopium*, *P. oxalicum*, *P. puberulum*	Saito et al. (1974) Purchio (1976) Tung et al. (1967) Udagawa et al. (1970)
Soy bean sauce (paste)	*A. flavus*, *A. parasiticus*	Sripathomswat and Thasnakorn (1981), Park et al. (1988)
Soy sauce (shoyu, jiunyu)	*A. flavus*, *P. citrinin*, *P. oxalicum* *P. variabile*	Saito et al. (1974), Tung et al. (1967), Udagawa et al. (1970)
Tien-mein-jiun (sweet flour paste)	*A.flavus*, *A. versicolor*	Zhang (1986)
Tsukemono	*P. cyclopium*, *P. puberulum*	Udagawa et al. (1970)

According to the study on the production of aflatoxins by *A. parasiticus* during the manufacture of Korean soy paste (Doenjang) and soy sauce (Kanjang), Park et al. (2003) demonstrated that aflatoxin could be produced on meju (crushed Korean soybean cake) during fermentation when either a pure culture *A. parasiticus*

(method A) or a mixed culture containing *A. parasiticus* in combination with *A. oryzae* and *B. subtilis* (method B) was inoculated to the meju. Figure 2.2 represents the amounts of aflatoxins were produced by the mixed culture on meju during fermentation. AFG_1 production was highly stimulated though it degraded quickly, whereas AFB_1 synthesis was low. After 2 months of ripening in brine, the content of aflatoxins decreased to 10-20% of the original level of the toxins. The degradation rate of AFG_1 was much faster than that of AFB_1.

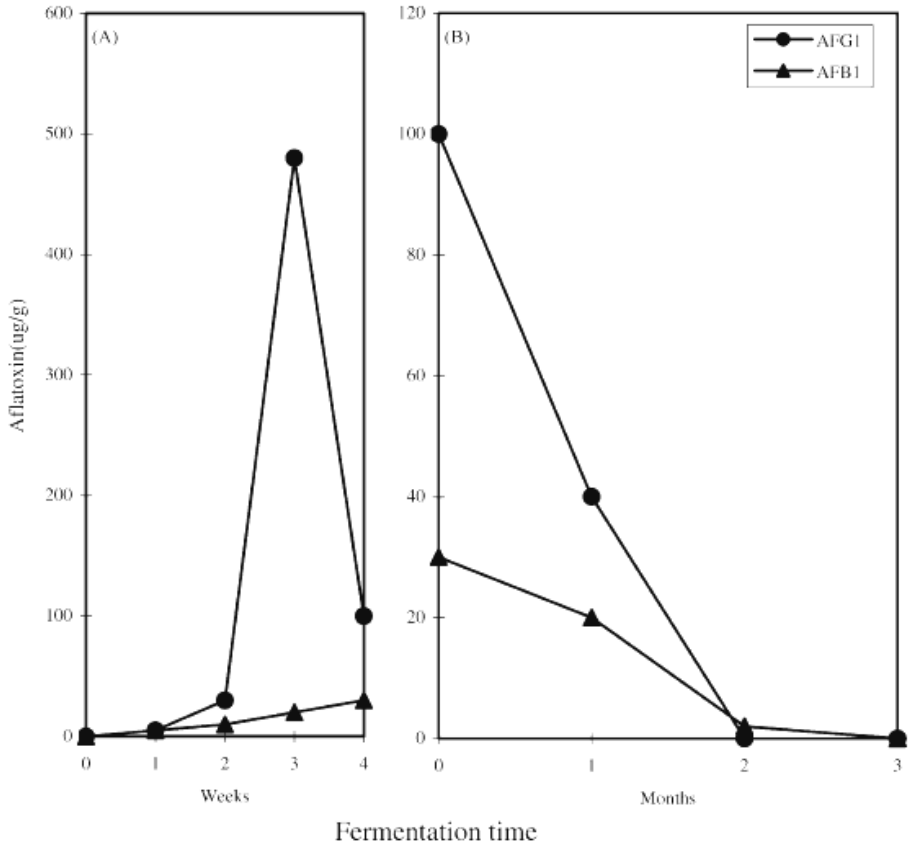

Fig. 2.2 Aflatoxin B_1 and G_1 production by *A. parasiticus* with *A. oryzae* and *B. subtilis* during the fermentation of meju.

It also cannot be assured that the soybean substrate does not support the production of other mycotoxins (Ciegler, 1975). For example, production of rubratoxin B in soybean has been demonstrated (Emeh and Marth, 1976). Therefore, wherever toxicogenic fungi are present in the commodities, under certain optimal conditions of moisture and temperature, there is always opportunity for the fungi to grow and to produce toxin.

In addition, the problem of possible of mycotoxins in the raw materials cannot be overlooked, as they have been found in a number of agricultural commodities around the world (Jelinek et al., 1989). In some areas, this might be a major source of contamination since poor grade grains are often used in fermentation. Numerous other surveys indicate that the presence of mycotoxins in the raw materials of food and feed is not uncommon (CAST, 1989). Because mycotoxins are low-molecular weight organic compounds with great stability, they may persist in contaminated commodities for years and, in most cases, cannot be completely removed during fermentation; that may thus be transmitted to the final product or to by-products.

2.4.4 Methods of aflatoxin decontamination

Foodstuffs distributed to the population should not represent a potential health hazard; therefore detoxification of mycotoxin-contaminated product has been a continuing challenge for the food industry. A great deal of concern has been directed towards aflatoxins because of their potency and ubiquity. Most of the factors obtained from studies on aflatoxins can be applied to other mycotoxins.

The sensitivity of mycotoxins to physical or chemical treatment is affected by many factors, including moisture content, location of the toxin in the food, forms of

the food, storage conditions, and interactions of the toxins with food components. It is important to understand these factors before a specific method can be recommended. In addition, the use of any applicable treatment conditions should not cause undesirable alteration of food quality (Samarajeewa et al., 1991). Mishra and Chitrangada (2003) proposed a review of some commonly used physical, chemical and biological methods of aflatoxin (Table 2.7).

Table 2.7 Some commonly used physical, chemical and biological methods of aflatoxin (Mishra and Chitrangada, 2003).

Methods	Conditions	Destruction (%)	Comments
Physical			Not very much effective and dereriorates organoleptic qualities in most of the cases. No single process can detoxify the entire toxin in liquid as well as solid infected commodity.
Sunlight	Bright sunlight effective on liquid commodity	99	
UV light	Chloroform solution of ground nut oil	45	
Microwave	6 KW, 4 min	95	
Autoclaving	121°C, 4 h	95	
Cooking	Steaming/ puffing	50	
Roasting	180°C, 30 min	80	
Pasteurization	80°C, 45 sec	64	
Dry heat	250°C	Partial	
Solvents	Specific for each solvents	80-95	

Table 2.7 Continued.

Methods	Conditions	Destruction (%)	Comments
Chemical			Harmful chemical residues may be left after the detoxification and also more toxic compounds may be generated by the reaction process.
H_2O_2	6% H_2O_2, 30 min, 80°C, alkaline pH	97	
Ozone	2 h, 100°C, 22% moisture	100	
Ammonia	40 psig, 100°C, 4%NH_4OH, 30 min	99	
Urea+Urease	20% Urea, 2% soyflour, sunlight 14 h	85	
Na-hypochlorite	15 mg Cl_2 gas per 100 mg AFB_1	100	
Na-bisulphite	0.4%, 5 min	45	
Biological			It is expensive, if breeding of resistant varieties are tried they are time consuming and also infection of some more fungi and bacteria are not expected
F.aurantiacum NRRL B184	Aqueous reaction medium	100	
Tetrahymena Pyriformis	Liquid reaction medium	Partial	
Bacillus spp.			
Rhizopus spp.			

Some others data about decontamination methods can be found in letterature and are presented below.

2.4.4.1 Physical methods

Among physical methods one can find:

- Well-selected harvesting, storage and processing methods
- Manual selection
- Dry-cleaning

Ohers alternative methods have also been proposed

2.4.6.1.1 Thermal inactivation

Aflatoxins are resistant to thermal inactivation and are not destroyed completely by boiling water, autoclaving, or a variety of food and feed processing procedure (Christensen et al., 1977). Aflatoxins may be destroyed partially by conventional processing procedures such as oil and dry roasting of peanuts to be used as salted nuts, in confections, or in peanut butter. Lee and co-workers (1969) reported a 45-83% reduction in aflatoxin that was dependent on roasting conditions and initial aflatoxin concentrations in raw peanuts. Comparable results were obtained in an investigation by Waltking (1971). In other studies, roasting conditions resulted in a significant decrease in the aflatoxin content of nuts, oilseed meals (Escher et al., 1973), and corn (Conway et al., 1978). The degradation of aflatoxins was a direct function of temperature, heating interval, and moisture content (Mann et al., 1967). Only partial destruction of aflatoxins in contaminated wheat occurs during the various

stages of bread making (Jemmali and Lafont, 1972). Baking temperatures do not significantly alter the levels of aflatoxin in dough (Reiss, 1978).

A range of reduction (32-87%) in the level of aflatoxin M_1 in freeze-dried milk (resulting from pasteurization, sterilization, preparation of evaporated milk, roller drying, and spray drying) was reported by Purchase et al. (1972). Detoxification was confirmed by the duckling bioassay. Also, aflatoxins were not detected in cottage cheese (prepared from the contaminated milk), but were present in the whey. In other studies, aflatoxin M_1 was apparently stable in raw milk and was resistant to pasteurization and processing (Stoloff et al., 1975).

A considerable reduction in aflatoxin levels has been associated with the limewater treatment (nixtamalization) of corn to produce tortillas. However, subsequent studies have shown that much of the original aflatoxin is reformed on acidification of the products (Price and Jorgensen, 1985). Comparable results for aflatoxins B_1, B_2, G_1, and G_2 have been reported by Bailey et al. (1990, 1991). Lime (regenerating calcium hydroxide in water) was postulated to react with aflatoxin, resulting in (1) a loss of indigenous fluorescence and (2) a major change in the extractability of aflatoxin in solvents such as chloroform. Evidence suggests that aflatoxin may be “masked” chemically in alkaline-processed corn (i.e., tortillas and corn chips) and, thus, may escape analytical detection. More importantly, reformation of parent aflatoxin may occur under the acidic conditions that are found in the stomach (Martinez, 1979).

Specific criteria have been established for evaluating the acceptance of a given aflatoxin reduction or decontamination procedure. The process must: (1) inactivate, destroy, or remove the toxin; (2) not produce or leave toxic residues in food; (3) retain

nutritive value and feed acceptability of the product; and, if possible, (5) destroy fungal spores (Park et al., 1988). Although some destruction of aflatoxins in feed-grade meals has been achieved by conventional processing procedures, heat and moisture alone do not provide a very effective method of detoxification.

2.4.4.1.2 Irradiation

Studies have demonstrated that exposures of contaminate peanut oil to shortwave and longwave UV light causes a marked reduction in the concentration of aflatoxins (Shantha and Sreenivasa, 1977). A 14-h exposure to sunlight destroyed approximately 77-90% of aflatoxin B_1 that was added to groundnut flakes, whereas only 50% of the toxin was destroyed in naturally contaminated product (Shantha et al., 1986). Gamma radiation did not result in degradation of aflatoxin in contaminated peanut meal, and UV light produced no observable change in fluorescence or toxicity of the treated sample (Feuell, 1966). Exposure of contaminated milk to UV light for 20 min at 25°C decreased the aflatoxin M_1 content by 89.1% in the presence of 0.05% peroxide, compared with 60.7% in peroxide-free milk (Yousef and Marth, 1986). Aflatoxins produce singlet oxygen after exposure to UV light and singlet oxygen further activates these chemicals to mutagens. DNA photobinding and mutagenesis by aflatoxins were enhanced by aflatoxin B_2 in a synergistic manner. Interestingly, these findings imply that aflatoxin B_2 (which often is found in combination with aflatoxin B_1) may amplify the activation of aflatoxin B_1 by sunlight (Stark et al., 1990). Jorgensen and co-workers (1992) found comparative mutagenic activity for equal concentrations of aflatoxin B_1 and mixtures of aflatoxin B_1 and B_2 using the *Salmonella* assay.

2.4.4.1.3 Solvent extraction and mechanical separation

Several suitable solvent systems are capable of extracting aflatoxins from different commodities with minimal effects on protein content or nutritional quality (Rayner et al., 1977). Theses systems include 95% ethanol, 90% aqueous acetone, 80% isopropanol, hexane-ethanol, hexane-methanol, hexane-acetone-water, and hexane-ethanol-water. However, current extraction technology for the detoxification of aflatoxin-contaminated oilseed meals appears to be impractical and cost prohibitive (Shantha, 1987). Most of the aflatoxin associated with contaminated corn or peanuts can be found in a relatively small number of kernels or seeds, providing and excellent opportunity to reduce the level of aflatoxin contamination by a variety of separation approaches.

Conventional methods used to clean corn (e.g. dry cleaning, wet cleaning, density separation, and preferential fragmentation) are somewhat effective in reducing the aflatoxin content (Brekke et al., 1975). Milling of corn resulted in better results. The distribution of aflatoxins was apparently low in grits and high in the germ, hull, or degermer fines of dry milled corn (Brekke et al., 1975). Aflatoxin occurred mainly in the steepwater and fiber of wet milled corn, with smaller amounts present in the gluten and germ (Yahl et al., 1971).

2.4.4.1.4 Density segregation

A novel method has been reported for reducing the aflatoxin concentration in corn (Huff and Hagler, 1982) and peanuts (Kirksey et al., 1987) by flotation and density segregation of toxic kernels. In a study by Kirksey et al. (1989), 95% of the aflatoxin in 21 of 29 samples of peanuts was contained in kernels that floated in tap

water. The mean aflatoxin level was decreased from 301 ppb to 20 ppb using this procedure. Flotation for whole-kernel grain and shelled unblanched peanuts should be compatible with current methods for wet milling or alkaline processing of corn (Hagler, 1991).

2.4.4.1.5 Adsorption from solution

Adsorbent materials including activated carbon (Decker, 1980) and clay and zeolitic minerals (Masimanco et al., 1973) have been shown to bind and remove aflatoxins from aqueous solutions such as water, Sorensen buffer, Czapek's medium, Pilsner beer, sorghum beer, whole milk, and skimmed milk. The adsorption of aflatoxins by clay (which is incorporated to remove pigments from crude oils) is a major factor resulting in the significant reduction of aflatoxins in refined peanut and corn oils. Reports indicate that hyllosilicate clay effectively removes aflatoxins from contaminated peanut oil and prevents its mutagenicity and toxicity *in vitro*. Ellis and co-workers (1990, 1991) reported that HSCAS, at levels as low as 0.5% (w/w), resulted in significant removal of aflatoxin M_1 from naturally contaminated skim milk, with negligible change in nutritional quality (i.e., total milk solids, lactose, and protein) as determined by proximate analysis (Figure 2.3).

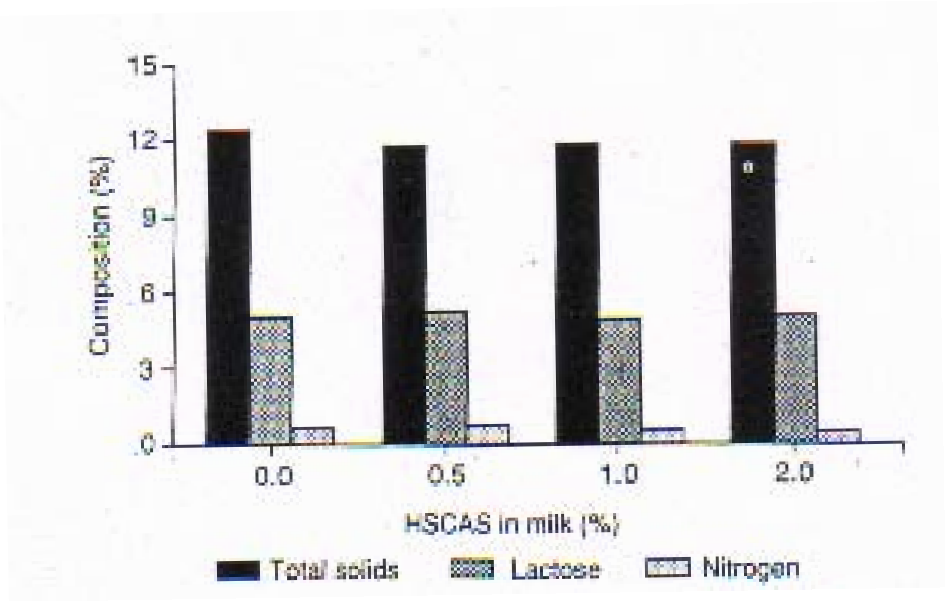

Fig. 2.3 Proximate analysis of nutritional components from HSCAS-treated skim milk that was contaminated naturally with aflatoxin M_1 at 0.75 ppb.

2.4.4.2 Biological methods

Established techniques for the management of aflatoxin contamination during crop production have not adequately insured aflatoxin-free commodities prior to harvest. A new pre-harvest strategy for the prevention of aflatoxin contamination of peanuts and cottonseeds is the use of nontoxigenic strains of *A. parasiticus* and *A. flavus* fungi to complete with (and exclude) toxin producing strains. Initial studies have shown that bio-prevention can be used to reduce pre-harvest aflatoxin contamination in peanuts and cottonseed significantly. Additional studies are justified to address human health and environmental impact concerns of this technology.

A promising new approach to the prevention (or elimination) of pre-harvest aflatoxin contamination is the molecular regulation of aflatoxin biosynthesis by toxigenic *Aspergillus* fungi. Enzymes that govern multiple steps in the biosynthetic

pathway of aflatoxin have been purified (Bhatnagar et al., 1989), providing investigators with the key to characterizing aflatoxin pathway genes and their regulation at the molecular level (Cleveland and Bhatnagar, 1991). A complete understanding of the molecular regulation of aflatoxin biosynthesis may lead to enhance food safety through selection of crops that minimize expression of aflatoxin pathway gene (Cleveland and Bhatnagar, 1991).

2.4.4.2.1 Enzymatic degradation or adhesion of aflatoxin by bacteria

Microorganisms (including yeasts, mold, and bacteria) have been screened for their ability to modify or inactivate aflatoxins. *Flavobacterium aurantiacum* (NRRL B-184) was shown to remove aflatoxin from a liquid medium significantly without the production of toxic by-products (Ciegler et al., 1966). Temperature and pH influenced the uptake of the toxin by the cells. A high population of cells (10^{11} cells/ml) permanently removed greater percentages of the aflatoxin from solution irreversibly than did lower populations. The rates of removal of AFB_1 by live cells were much higher compared with dead cells, but both these cell types adsorbed some amount of AFB_1. Also, the release of labeled CO_2 only by the living cells shows that some amount of AFB_1 is metabolized flabacteria cells. Copper and Zinc ions inhibit the degradation of aflatoxin. These effects show the influence of the enzyme in the degradation process (D'Souza and Brackett, 2001). Hence, the possible mechanisms involve with Proteinase-K enzyme and it is not due to nonspecific binding with the bacterium's genome (Smiley and Droughon, 2000). Efficient degradation occurred at pH 7.0.

The same investigators also found that certain acid-producing molds could catalyze the hydration of aflatoxin B_1 to B_{2a} (a less toxic product). The applications of microbial detoxification of aflatoxins have been reviewed (Ciegler, 1978). Hao and co-workers (1987) reported that *F. aurantiacum* removed aflatoxin B_1 from peanut milk. This bacterium grew in both defatted and partially defatted peanut milk and was not inhibited by the presence of aflatoxin.

2.4.4.2.2 Binding of aflatoxin by some probiotic bacterial strains

Karunaratne (1990) showed that *Lactobacillus acidophilus, L. bulgaricus,* and *L. planatarum* could be used to either prevent mold growth or to remove aflatoxin. The effect of AFB_1 adhesion capability of *Lactobacillus rhamnosus* strain GG was investigated by Kankaanpaa and Tuomola (2000) using a caco-2 adhesion model. The removal of AFB_1 by the strain reduced its adhesion capability from 30 to 5%. It was therefore concluded by them that aflatoxins may influence the adhesion properties of probiotic bacteria able to sequester them, and subsequently it may reduce the accumulation of AFB_1 in the intestine via increased aflatoxin-bacteria complex. Nezami et al. (2000) studied the adhesion capability of *L. rhamnosus* strain GG and LC 705 and *Propionibacterium freudenrichhi* spp. *shermani* JS with aflatoxin. The complexs formed *in vitro* with AFB_1 and *lactobacilli* strains were stable for 1 h. The binding efficiency of AFB_1 at late exponential and early stationary phase by these bacteria by pronase E, lipase, m-periodate were consistent; the effect of urea suggested hydrophobic interaction and the effect of NaCl and $CaCl_2$ proved electrostatic interaction, while a pH profile suggested that the interaction may involve hydrogen bonding as well. Viable, heat-killed, and acid-killed bacteria responded in a

similar manner (Haskard et al., 2000). *Bifidobacterium* also can bind to AFB_1 efficiently (Peltonen et al., 2000).

2.4.4.2.3 Biocompetitive inhibition of aflatoxin by bacteria

In 1995, H.K. Chourasia published his experimental results on kernel infection and aflatoxin production in peanut (*Arachis hypogaea* L) by *A. flavus* in the presence of geocarposporic bacteria. He hypothesized that geocarposporic bacteria would be ideal for protecting the developing groundnut pods against aflatoxigenic fungi. *A. flavus* was grown on groundnut extract agar and on viable groundnut kernels, either in pure culture or in dual culture with either of *Bacillus megaterium, B. laterosporus, Cellulomonas cartae, F. odoratum, Phyllobacterium rubiacearum, Pseudomonas aurofaciens*, and *Xanthomonas maltophia*. Aflatoxin production by *A. flavus*, its growth, and interactions with other microorganisms were studied, and it was concluded that *F. odoratum* showed inhibition in aflatoxin biosynthesis.

2.4.4.2.4 Control by fungi or bacteria

Detroy and Hesseltine (1969) studied the conversion of AFB_1 to aflatoxicol by steroid metabolizing fungi. However, the process was very slow and incomplete (60% detoxification). *Tetrahymena pyriformis* W decreased the concentration of 2 µg AFB_1/ml by 67% in 48 h with production of a biologically reduced AFB_1 (Robertson et al., 1970) as shown in Figure 2.4. The carbonyl in cyclopentane ring of AFB_1 was reduced to a hydroxyl. Tsubouchi et al. (1980) studied degradation of AFB_1 by *Aspergillus niger*. Interconversion of AFB_1 and aflatoxicol by several fungi was studied by Nakazato et al. (1990). They screened four fungal strains; *A. niger,*

Eurotium herbariorum, a *Rhizopus* spp. and nonaflatoxigenic *A. flavus* that could convert AFB_1 to aflatoxicol. They concluded that the interconversion occurred due to intracellular enzyme (cytochrome P-450 mono-oxygenases) of *A. flavus* and *Rhizopus* spp. Figure 2.5 shows the probable mechanism of AFB_1 degradation.

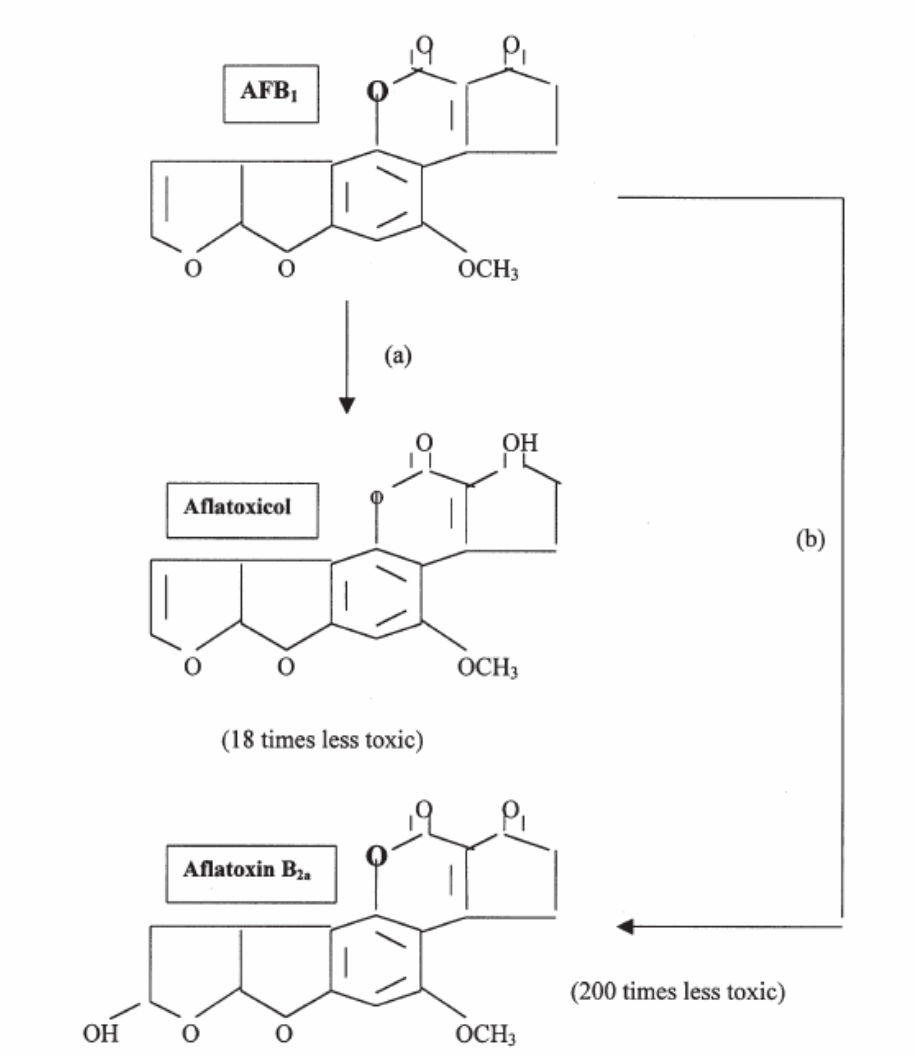

Fig. 2.4 (a) Biologically reduced AFB_1 by *T. pyriformis, Rhizopus* spp. (b) Hydroxy derivatives of AFB_1 by *Lactobacillus delbrueckii*.

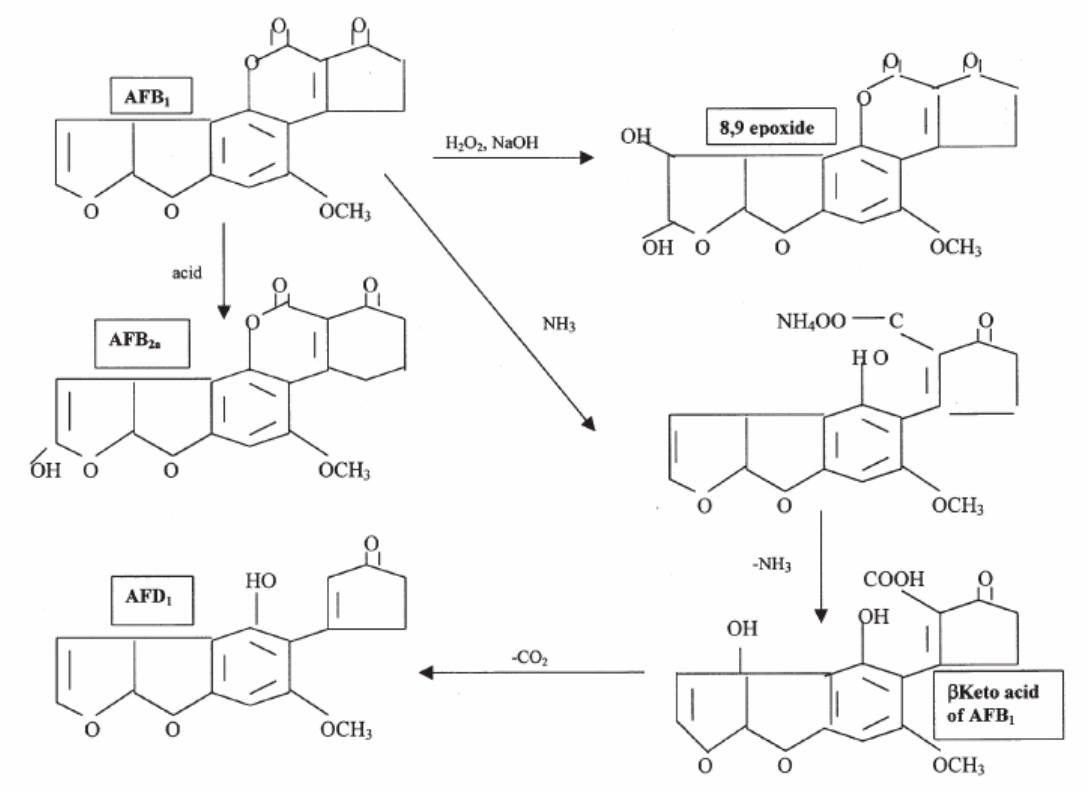

Fig. 2.5 Probable mechanism of degradation of aflatoxin B_1.

Munimbazi and Bullerman (1998) reported the influence of 6 *Bacillus pumilus* isolates on the growth of aflatoxin-producing molds and aflatoxin production using both simultaneous and deferred antagonism assays. Totally, percentages of inhibition of aflatoxin production ranged between 98.2% and 99%. Mycelium production was less inhibited with percentages of inhibition ranging between 34.4% and 56.4%. Bottone and Peluso (2003) identified a compound produced by *B. pumilus* which could inhibit *Aspergillus* species. The active compound inhibited *Aspergillus* spore germination and aborted elongating hyphae, presumably by inducing a cell-wall lesion.

The inoculation of *A. flavus* spores into a culture of *Streptococcus lactis* in tryptone broth medium resulted in little or no aflatoxin accumulation even though the growth of the fungus was not hindered. The drop in pH and reduced nutrient levels in the medium as a result of the *S. lactis* growth were not cause of the observed inhibition. The inhibition was not eliminated by the addition of carbohydrate equal to the amount used by the bacterium before the inoculation with the fungus. Aflatoxin levels were also markedly reduced when *S. lactis* was inoculated into a growing *A. flavus* culture. In addition to inhibiting the synthesis of aflatoxin, *S. lactis* also degraded preformed toxin. *S. lactis* produced and excreted the inhibitor into the medium late in its growth phase. The inhibition was a heat-stable low-molecular-weight compound. Wiseman and Marth (1981) also found that *S. actis* had the ability to inhibit aflatoxin.

Finally, fermentation of contaminated grains has been shown to degrade aflatoxins (Dam et al., 1977), but ensiling aflatoxin-contaminated high-moisture corn was apparently not as effective. This difference may be distributed to the production of insufficient acid to catalyze the transformation of aflatoxin B_1 to aflatoxin B_{2a} by this method (Lindenfelser and Ciegler, 1970).

2.4.4.3 Chemical methods

A diverse group of chemicals has been tested for the ability to degrade and inactivate aflatoxins. These chemicals include numerous acids, bases, aldehydes, bisulfite, oxidizing agents, and various gases. A number of these chemicals can react to destroy (or degrade) aflatoxins effectively but most are impractical or potentially unsafe because of the formation of toxic residues or the perturbation of nutrient

content, flavor, odor, color, texture, or functional properties of the product. Two chemical approaches to the detoxification of aflatoxins that have received considerable attention are ammoniation and reaction with sodium bisulfite.

2.4.4.3.1 Ammoniation

Treatment of grain with ammonia appears to be available approach to the detoxification of aflatoxins. Ammoniation (under appropriate conditions) results in a significant reduction in the level of aflatoxins in contaminated peanut and cottonseed meals (Dollear et al., 1968) and corn (Brekke et al., 1977). The ammoniation process, using ammonium hydroxide or gaseous ammonia, has been shown to reduce aflatoxin levels in corn, peanut-meal cakes, and whole cottonseed and cottonseed products by more than 99%. If the reaction is allowed to proceed sufficiently, the process is irreversible. Primarily, two procedures are used: a high pressure and temperature process (HP/HT) at feed mills or an atmospheric pressure and ambient temperature procedure (AP/AT) on the farm (Table 2.8).

Table 2.8 Parameters and application of ammoniation aflatoxin decontamination procedures.

Parameter	Process	
	High pressure/high temperature	Ambient pressure/atmospheric temperature
Ammonia level (%)	0.5-2	1-5
Pressure (PSI)	35-50	Atmospheric
Temperature (°C)	80-120	Ambient
Duration	20-60 min	14-42 day
Moisture (%)	12-16	12-16
Commodities	Whole cottonseed, cottonseed meal, peanut meal, corn	Whole cottonseed, corn
Application	Feed mill	Farm

The HP/HT process involves the treatment of the contaminated product with anhydrous ammonia and water in a contained vessel. The amount of ammonia (0.5-2%), moisture (12-16%), pressure (35-50 psi), time (20-60 min), and temperature (80-120%) varies with respect to the initial levels of aflatoxin in the product. The AP/AT process also uses a 13% ammonia solution, which is sprayed on the seed as it is packed into a plastic silage-type bag (approximately 10 feet diameter by 100 feet long). After this procedure, the bag is sealed and held at ambient temperature (25-45°C) for 14-12 days. The holding time will vary according to the ambient temperature, that is, a lower ambient temperature will require a longer holding time.

The amount of ammonia (1-5%), moisture (12-16%), and time (14-42 days) will vary according to the initial levels of aflatoxin present. With this process, the bag is probed and tested periodically until test results show that aflatoxin levels are equal to or below 20 ppb.

The safety of ammoniated corn has been evaluated in rainbow trout, chickens, and rats. The finding results provide strong evidence that chemical treatment via ammoniation can provide an effective strategy to detoxify aflatoxin-contaminated crops.

The mechanism for this action appears to involve hydrolysis of the lactone ring and chemical conversion of the parent compound aflatoxin B_1 to numerous products that exhibit greatly decreased toxicity. Two major products (Figure 2.6), identified as compounds with molecular weight 286 (aflatoxin D_1) and molecular weight 206, have been isolated and tested in various biological systems (Park et al., 1988). Reaction products of ammoniation are dependent on temperature, pressure, and the source of ammonia.

The use of aqueous or gaseous ammonia, as well as other chemical treatments for the detoxification of aflatoxins, has been review thoroughly. The ammoniation method is widely used in United State and Mexico. In addition, it is used routinely in France, South Africa, Senegal, and Brazil and soon will be utilized in the Sudan and India to lower aflatoxin contamination levels. Several member countries of the European Economic Community import ammonia-treated peanut meal on a regular basis (Park and Lee, 1990).

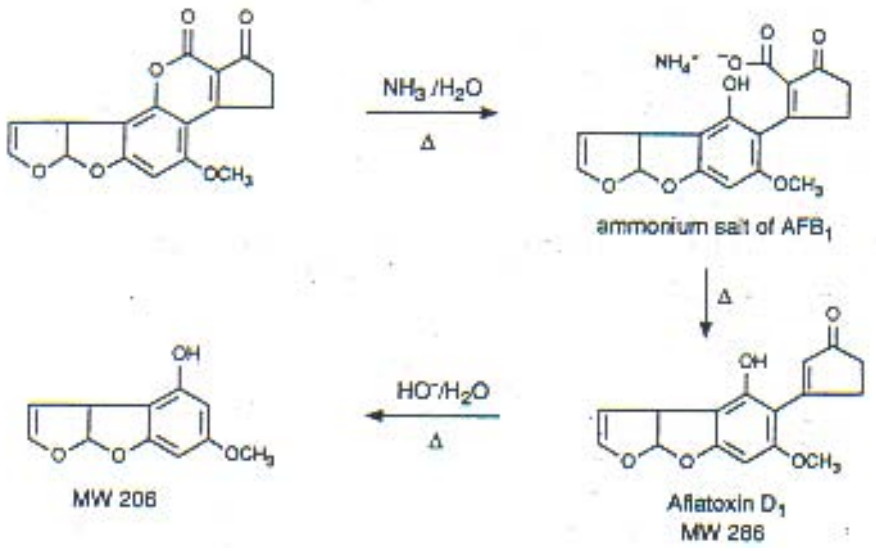

Fig. 2.6 Proposed major reaction products from the ammoniation of aflatoxin B_1.

In fact, ammonia is a toxic chemical, so you must have proper equipment in order to apply in a controlled safe manner. Ammonia can be deadly when used improperly. Always follow safety precautions. Ammonia's density is lower than air density. If a large amount of ammonia is released to the air, people should rapidly leave the polluted area, moving into the wind. When driving out air from an ammonia container, the gas outlet valve should be in a low position. Ammonia gas can irritate the eyes. Prolonged inhalation can cause suffocation and death. Anhydrous ammonia also can cause freeze burn on the skin because it is both caustic and has a low boiling point. A large supply of water should be close at hand for flushing eyes or skin if an accident occurs. When handling anhydrous ammonia, always wear goggles, gloves and a long-sleeved shirt. Avoid breathing ammonia vapors. Never allow a spark, flame or electrical equipment near the ammonia.

Ammonia is corrosive to metal (copper and brass fittings cannot be used) and reacts with galvanized metal, making ammonia very corrosive to grain bins. Ammonia cannot be forced into a grain-filled bin with a drying or aerating fan. Not only is the practice dangerous and destructive, but the ammonia readily escapes and has little benefit in detoxifying corn.

When handling corn with high levels of aflatoxin, always wear a dust mask. The contaminated dust is hazardous. Where dust is a major problem, as in grain cleaning, a self-contained respirator should be worn.

2.4.4.3.2 Treatment with Bisulfite

Sodium bisulfite has been shown to react with aflatoxins (B_1, G_1, M_1, and aflatoxicol) under various conditions of temperature, concentration, and time to form water-soluble products.

Several studies indicate that peroxide and heat enhance the destruction of aflatoxin B_1 by sodium bisulfite added to dried figs (Altug et al., 1990). Clearly more research is warranted in this area to determine the potential of bisulfites to reduce or inactivate aflatoxins in food and feed (Hagler, 1991). However, sulfur dioxide can be harmful for some sensible persons so the concentration in food is regulated.

2.4.4.3.3 Heterogeneous catalytic degradation

The formation of aflatoxin B_1 adsorption complexes on the surfaces of certain inorganic materials (including various alumina, silica, aluminosilicate, and chemically modified aluminosilicate) may promote heterogeneous catalytic degradation of the parent molecule after desorption. Phillip and co-workers (1988) observed that organic

extracts from reactions of aflatoxin B_1 with alumina, zeolite, and phyllosilicate contained varying levels of aflatoxin degradation products including aflatoxin B_2, which was identified as the major product in most casts (Figure 2.7). The parent aflatoxin that was reacted with activated charcoal was recovered from the complex unchanged. Perhaps chemical degradation of aflatoxins by reactive inorganic adsorbents represents another useful chemical approach to detoxification.

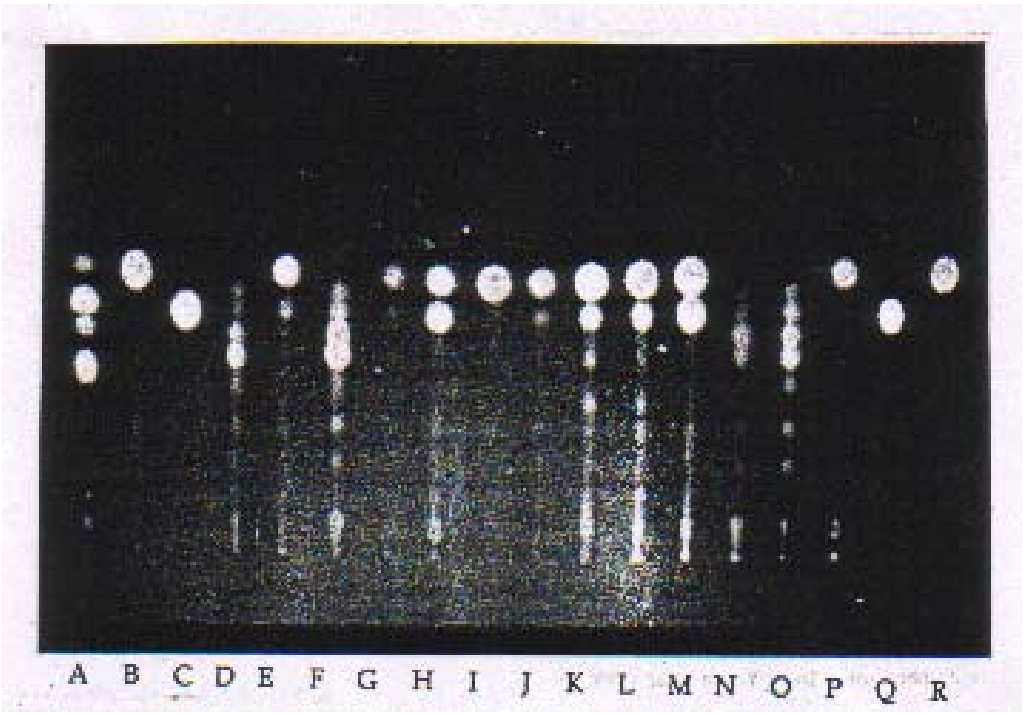

Fig. 2.7 TLC of 18 pellet extracts from aflatoxin-treated samples.

2.4.4.3.4 Reduction in bioavailable aflatoxin by selective chemisorption

A phyllosilicate clay (HSCAS or NovaSil™) currently available as an anticaking agent for animal feeds has been reported to (1) tightly bind aflatoxins in aqueous suspension (Figure 2.8); (2) markedly diminish aflatoxin uptake by the blood and distribution to target organs (Figure 2.9); (3) prevent aflatoxicosis in farm animals, including chickens (Figure 2.10), turkey, poults, goats, pigs, and mink; and

(4) decrease the level of aflatoxin M_1 residues in milk from lactating dairy cattle. Consequently, HSCAS effectively removed aflatoxin M_1 from contaminated skim milk.

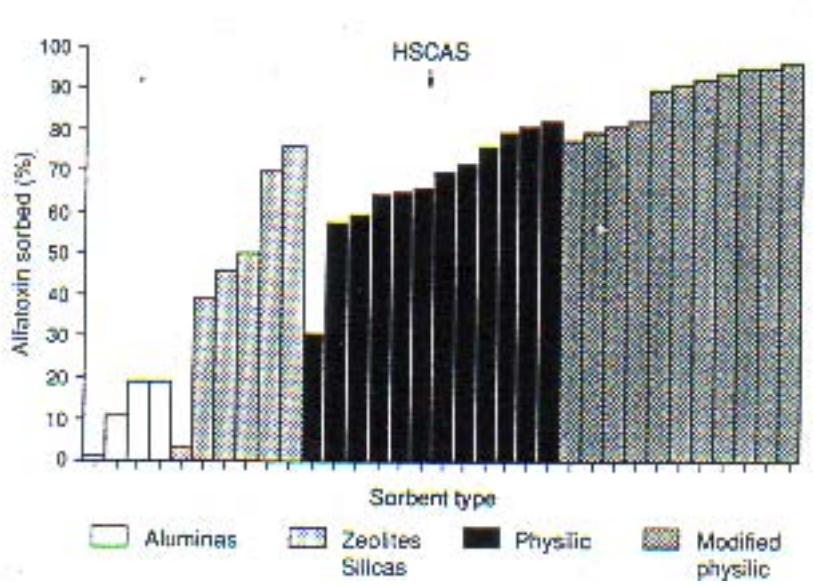

Fig. 2.8 Binding of radiolabeled aflatoxin B_1 by alumina, zeolite, silica, phyllosilicate, and chemically modified phyllosilicates.

Phyllosillicates are layer-lattice silicate and chain silicate that are essentially composed of repeating layers (or chains) or (1) divalent or trivalent cation (e.g. alumina) held in octahedral coordination with oxygen and hydroxyl and (2) silica that are coordinated tetrahedrally with oxygen and hydroxyl. Condensation of layers in a 1:1 ratio gives rise to dimorphic phyllosilicate clays with the general formula $M_{2\text{-}3}Si_2O_5(OH)_4$. Trimorphic phyllosilicate are formed by a 1:2 condensation of layers with (octahedrally coordinated aluminium sandwiched between two layers of tetrahedrally coordinated silica) with the formula $M_{2\text{-}3}Si_4O_{10}(OH)_2$. Chain phyllosilicates are composed of trimorphic layers arranged in chains (or bands) that are joined through oxygen ions (Schulze, 1989). Generally, these materials posses

three types of binding sites for aflatoxins: (1) those located within interlayer channels, (2) those located on the surface, and (3) uncoordinated metal ions located at the edges of HSCAS particles.

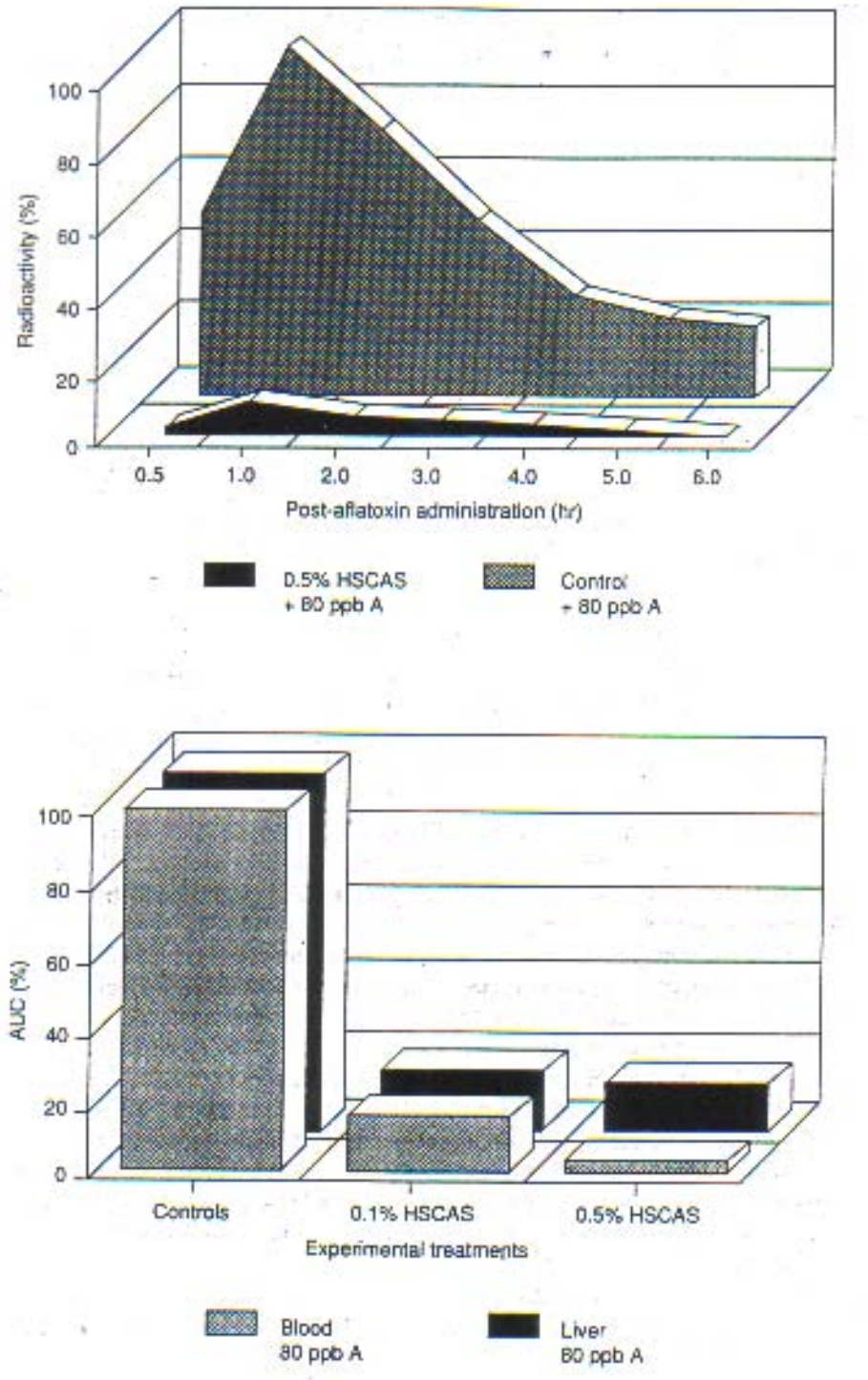

Fig. 2.9 (*Top*) [^{14}C] Aflatoxin B_1 in the blood of broiler chicks fed the equivalent of 80 ppb aflatoxin with (*front*) and without (*back*) 0.5% HSCAS is their diet. (*Bottom*) Relative bioavailability of [^{14}C] aflatoxin B_1 in the blood (hatched) and liver (solid) of

chicks fed the equivalent of 80 ppb total aflatoxin with and without 0.1% and 0.5% HSCAS in their diet.

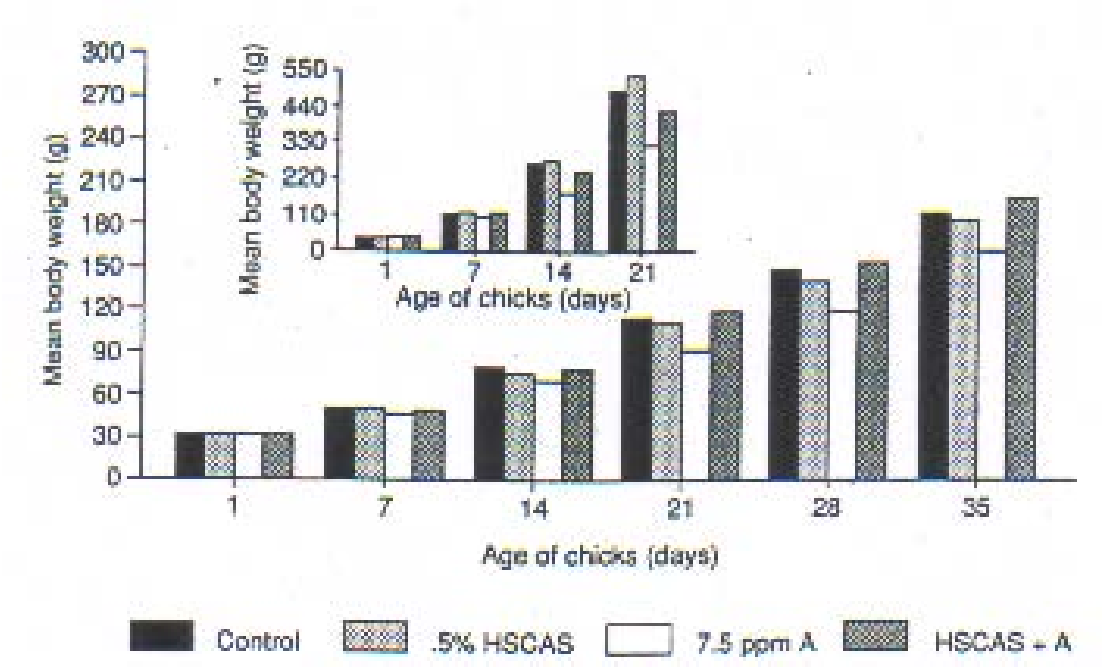

Fig. 2.10 Protective effects of HSCAS in broiler (inset) and Leghorn chicks fed AFB_1-contaminated diets.

2.4.5 International legislation for aflatoxins

The hazardous nature of aflatoxin to humans and animals has necessitated the need for establishment of control measures and tolerance levels by national and international authorities. Different countries have different regulations for aflatoxin. The general trend is that industrialized countries usually set lower tolerance levels than the developing countries, where most of the susceptible commodities are produced. For example, the tolerance level for aflatoxin in food is 5 µg/kg in Sweden (Akerstrand and Möller, 1989) and 10µg/kg in Japan (Aibara and Maeda, 1989),

whereas it is 30 µg/kg in Brazil (Sabino et al., 1989a). However, such lack of harmony may give rise to difficulties in the trade of some commodities.

The first legislative act was undertaken in 1965 by the Food and Drug Administration (FDA) of the USA, which proposed a tolerance level of 30 µg/kg of total aflatoxins (B_1+G_1+B_2+G_2). With increasing awareness of aflatoxins as potent toxic substance, the proposed level was lowered to 20 µg/kg in 1969 (FDA, 1977). Since then, the tolerance level have been revised and re-evaluated occasionally. The current tolerance levels established by the FDA for food and feeds are listed in Table 2.9.

Table 2.9 FDA tolerance levels for total aflatoxin.

Item	Tolerance level (µg/kg)
Food for human consumption	20
Feed for beef cattle and poultry	300
Feed for swine	200
Feed for breeding livestock	100
Feed for dairy cattle	20
Milk	0.5

Source: Park & Nijapau (1989); Park & Liang (1993)

In 1973, the European Economic Community (EEC) established legislation on maximum permitted levels of AFB_1 in different types of feedstuffs (EEC, 1974). The legislation has been frequently amended since then. However, the main framework of the legislation remains more or less the same. Table 2.10 lists the maximum allowable

levels of AFB_1 in animal feeds as established by the EEC. The legislation has been in action since 30 November 1991 (EEC, 1991).

In 1998 the European Union (EU) established maximum allowable limits for aflatoxin B (AFB_1) and total aflatoxins (AFLs) in peanuts (2 μg/kg for AFB_1 and 4 μg/kg for AFLs in peanuts intended for human consumption; 8 μg/kg for AFB_1 and 15 μg/kg for AFLs in peanuts intended for further processing) and in cereals intended for human consumption or as an ingredient (2 μg/kg for AFB_1 and 4 μg/kg for AFLs). Limits were also established for aflatoxin M_1 (AFM_1) in milk and milk products (0.05 μg/kg), and limits for others mycotoxins are currently under discussion.

Contamination of agricultural commodities with aflatoxin, and hence establishment of regulations, has been a major concern to the Joint FAO/WHO Expert Committee. The Committee recommends that the presence of aflatoxin in food should be limited to “irreducible levels”. An irreducible level is defined as: “the concentration of a substance that can not be eliminated from a food without involving the discarding of that food altogether or severely compromising the ultimate availability of major food supplier” (FAO/WHO, 1987). The current regulations for aflatoxin established by the Joint FAO/WHO Committee are given in Table 2.11.

Table 2.10 Maximum permitted level of aflatoxin B_1 in different animal feeds established by the EEC[a].

Feed	Level (µg/kg)[b]
Straight feeds[c]	
Peanut, copra, palm kernels, cotton seed, babassu, maize and product derived thereform	20
Compound feeds[d]	
Complete feeds	
Feeds for cattle, sheep and goat (except dairy cattle, calves and lambs)	50
Feeds for pig and poultry (except young animals)	20
Other complete feeds	10
Complementary feeds	
Feeds for cattle, sheep and goats (with exception of dairy animals)	50
Feeds for pigs and poultry (with exception of young animals)	30
Other complementary feeds	5

[a] EEC (1991).

[b] Based on feed moisture content 12%.

[c] Feed composed of a single item.

[d] Feed composed of a mixture items.

Table 2.11 Aflatoxin legislations set by the Joint FAO/WHO Expert Committee[a].

Aflatoxin	Tolerance level (µg/kg)	Food/Feed
B_1	5	Feed for dairy cattle
M_1	0.05	Milk
$B_1+G_1+B_2+G_2$	15	Raw peanut for human consumption
$B_1+G_1+B_2+G_2$	10	Processed peanut for human consumption

[a] FAO/WHO (1990, 1992)

The Dutch authorities undertook a worldwide enquiry, during 1986 and 1987, in which 66 countries were requested to report on their regulations and tolerance limits for mycotoxins in food and feeds. Of the 66 countries, 50 had enacted or proposed legislation for aflatoxins in foods, 35 for aflatoxins in feedstuffs, and 14 for aflatoxin M_1 in milk and dairy products.

2.5 Ochratoxin A

Ochratoxin A (CAS No. 303-47-9) is a toxic metabolite produced primarily by *Aspergillus* but also by *Penicillium* and other mold. There are three generally recognized ochratoxins, designated A, B and C. Ochratoxin A (OTA) is chlorinated and is the most toxic, followed by OTB and OTC. Only ochratoxin A, and very rarely its dechloro analog, ochratoxin B, have been found in foods and feeds, whereas in culture filtrates of the generating molds, the methyl and/or ethyl esters as well as the

4-hydroxy-derivative and the isocoumarion nucleus of ochratoxin B (ochratoxin α) have been observed (Visconti and Bottalico, 1983). These compound are produced by a variety of fungi, with the more frigophilic *Penicillia* (e.g., *Penicillium viridicatum*, *P. verrucosum*) being the primary producers in the cooler areas of the world (Krogh, 1987).

2.5.1 Chemistry

Biosynthetically the ochratoxins are pentaketides, consisting of a chlorinated dihydroisocoumarin moiety coupled to a 7-carboxyl group by an amide bond to one molecule of L-β-phenylalanine (Pohland et al., 1982), with the following properties:

White, odorless, crystalline solid

MP_{21}	168-173°C (90°C, benzene)
α_D	-46.8° (2650 μmol/L, $CHCl_3$)
Mol.Wt.	403.8
λ_{max}^{MeOH}	214 nm (ε_{max} 37,200),
	282 nm (ε_{max} 890),
	332 nm (ε_{max} 63,300)

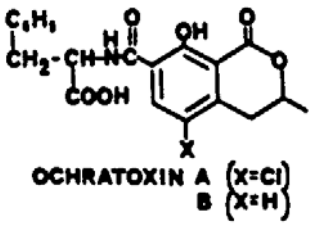

Fig 2.11 Structure of ochratoxin A.

OTA can be recrystallized from xylene, it forms crystals that emit green (acid solution) and blue (alkaline solution) fluorescence in ultraviolet light. The free acid of ochratoxin A is soluble in polar organic solvent (IARC V.31, 1983). The sodium salt is soluble in water. Ochratoxin A is unstable to light and air, degrading and fading even after brief exposure to light especially under humid conditions. Ethanol solutions are stable for longer than 1 year if kept in the dark and cold. OTA is fairly stable to heat; in cereal products, up to 35% of the toxin survives autoclaving for up to 3 hours. When heated to decomposition, the toxin emits toxic fumes of chlorine and NO_x. It is incompatible with strong oxidizing agents, strong acids, and strong bases.

2.5.2 Natural occurrence

Ochratoxin A is a naturally occurring mycotoxin. It exists completely in particulate phase in ambient atmosphere. It is immobile in soil. Its widespread occurrence in food and animal feed results in probable human exposure. OTA is a natural contaminant presented in several food products for example corn, peanuts, storage grains, cottonseed, and decaying vegetation (Merck, 1989). It has been detected in moldy cereals including wheat, maize, rye, barley, and oats; peanuts; coffee beans; bread; flour; rice; peas; and beans (IARC V.31, 1983). Cereals were found to be the main contributors (55%) to the total ochratoxin A intake (RIVM report, 2002).

Ochratoxin-producing fungi are included in the *Penicillium* and *Aspergillus* genera (IARC V.10, 1976). In warm regions, peanuts and soybeans are the main substrates for *Aspergillus ochraceus*. This fungus is found sporadically in a wide range of stored food commodities, including cereals, but is seldom the causes of

substantial concentrations of OTA. It may also infect coffee beans during sun-drying and is a source of OTA in green coffee beans. In the colder climates, ochratoxin A is formed most notably by *Penicillum* strains especially *Penicillium verrucosum* with maize, wheat and barley as the main substrates. Infection has been shown to occur pre-harvest, but post-harvest OTA formation is regarded as the predominant factor of OTA formation in food. As cereals are widely used in animal feeds, so this mycotoxin is also found in some animal product, especially pig kidney and liver.

A. carbonarius grows at high temperatures and is associated with maturing fruits, especially grapes. Because of its black spores, it is highly resistant to sunlight, and survives sun-drying. It is the source of OTA in fresh grapes, in dried vine fruits and wine; it is also one source of OTA in coffee.

The worldwide occurrence of ochratoxin A contamination of raw agricultural products (Table 2.12) has been amply documented. The natural occurrence of ochratoxin A in human foods has been also observed (Table 2.13).

Table 2.12 Worldwide occurrence of ochratoxin A in raw agricultural products. (Pohland et al., 1992)

Country	Commodity	Incidence (%)	Max.Level (ng/g)
Australia	Feeds	<1	70,000
Austria	Feeds	5-10	1,000
Canada	Feeds	<1	6,000
	Heated grains*	50-60	27,000
	Wheat, barley	<1	51
	Dried peas, beans	1-5	21
Czechoslovakia	Feeds	100	17
Denmark	Feeds*	50-60	27,500
	Barley malt	6	189
	Wheat, rye	1	50
Finland	Feeds	30-40	100
France	Corn	1-5	200
Germany (FRG)	Barley	10-20	206
	Corn	5-10	82
	Feeds	10-15	13
	Oats	10-15	59
	Wheat	5-10	137
Germany (GDR)	Corn	1-5	22
Hungary	Feeds	?	?

* Moldy

Table 2.12 Continued.

Country	Commodity	Incidence (%)	Max.Level (ng/g)
India	Grains	5-10	2,000
	Sorghum	5-10	?
Indonesia	Feed	?	500
Italy	Corn	40-50	1
Poland	Feed	1-5	200
	Grains	5	200
	Grains	?	3,000
	Rye	15-20	200
	Wheat	10-15	100
Sweden	Barley/Oats	5-10	410
Taiwan	Chicken feed	40-50	?
United Kingdom	Corn	30-40	500
	Feeds	1-5	250
	Oats	5-10	80
	Wheat/Barley	10-15	5,000
Yugoslavia	Corn	25-30	5,125
	Grains	60-70	68,900

* Moldy

Table 2.13 Worldwide occurrence of ochratoxin A in human foodstuffs. (Pohland et al., 1992)

Country	Commodity	Incidence (%)	Max.Level (ng/g)
Brazil	Cassava flour	<1	65
	Dried beans	<1	160
	Dried white corn	<1	32
Bulgaria *	Beans	17	27
**	Beans	7	50
*	Corn	27	35
**	Corn	9	25
Czechoslovakia	Cereals	5-10	17.5
France	Beer	10	110
Germany (GDR)	Coffee beans*	50	90
Germany (FRG)	Sausage	17.8	3.4
India	Cocoa products	<1	50
Italy	Bread*	?	80,000
	Green coffee	20-30	23
Japan	Rice*	?	430
	Green coffee	1-5	46
	Roasted coffee	5-10	17
Morocco	Olives/oil	1-5	80
Norway	Cereals	1-5	180

* Moldy

Table 2.13 Continued.

Country	Commodity	Incidence (%)	Max.Level (ng/g)
Poland	Cereals	5-10	1,200
	Flours	20-30	100
	Gruels	5-10	20
Sweden	Beans	5-10	442
	Peas	1-5	10
	Barley	1-5	11
Switzerland	Figs	10	160
	Flour (brown)	90	1.9
	Sausages	8	0.8
	Wheat products	90	3.5
Tunisia	Couscous		

* Moldy

Table 2.13 Continued.

Country	Commodity	Incidence (%)	Max.Level (ng/g)
United Kingdom	Bread*	40-50	80
	Cereals	10-15	108
	Cocoa beans, raw	18	500
	Cocoa beans, roasted	15.8	100
	Coffee, green	20-30	200
	Corn flour	30-40	200
	Corn oil	30	50
	Flour*	20-30	6,250
	Kidney (pork)	15.5	44
	Nuts	40	1
	Soya bean	36	500
	Soya flour	19	500
	Wheat flour	28	2,900
United States	Coffee beans (green)	5	360
Yugoslavia	Corn/Beans	40	5,000
	Beans	5-10	53
	Breads*	19	?

* Moldy

2.5.3 Adsorption, distribution, metabolism and excretion

2.5.3.1 General metabolism

2.5.3.1.1 Production of OTα

OTA is cleaved into Phe and OTα by peptidases in the intestinal mucosa, and in other organs such as pancreas as well as in vitro. OTα was previously regarded as harmless, but evidence for its genotoxicity has been recently found (Föllmann et al., 1995). Thus the level of OTα should also be determined in humans and in bigastric animals to assess the exposure.

2.5.3.1.2 Production of hydroxylated derivatives

In the presence of NADPH, liver microsomes from pigs, rats and humans metabolize OTA to produce hydroxylated derivatives (4R)- and (4S)-4-hydroxyochratoxin A (Oster et al., 1991). In the same conditions rabbit liver microsomes produce 10-hydroxyochratoxin A (Størmer et al., 1983).

OTA is also used as a substrate by Phe hydroxylase and transformed into tyrosine OTA (Tyr-OTA) (Creppy et al., 1990). This derivative is cytotoxic (Creppy et al., 1983b).

The P450 involved in the hydroxylation were, for the rat, CYP1A2 for the (4R)-isomer and CYP2B1 for the (4S)-isomer. In the pig microsomes the implicated cytochromes were CYP2B and CYP2C11 classified A2 and A3. One OTA metabolite more lipophilic than the parent compound has also been identified in pig liver microsomal incubations (Oster et al., 1991).

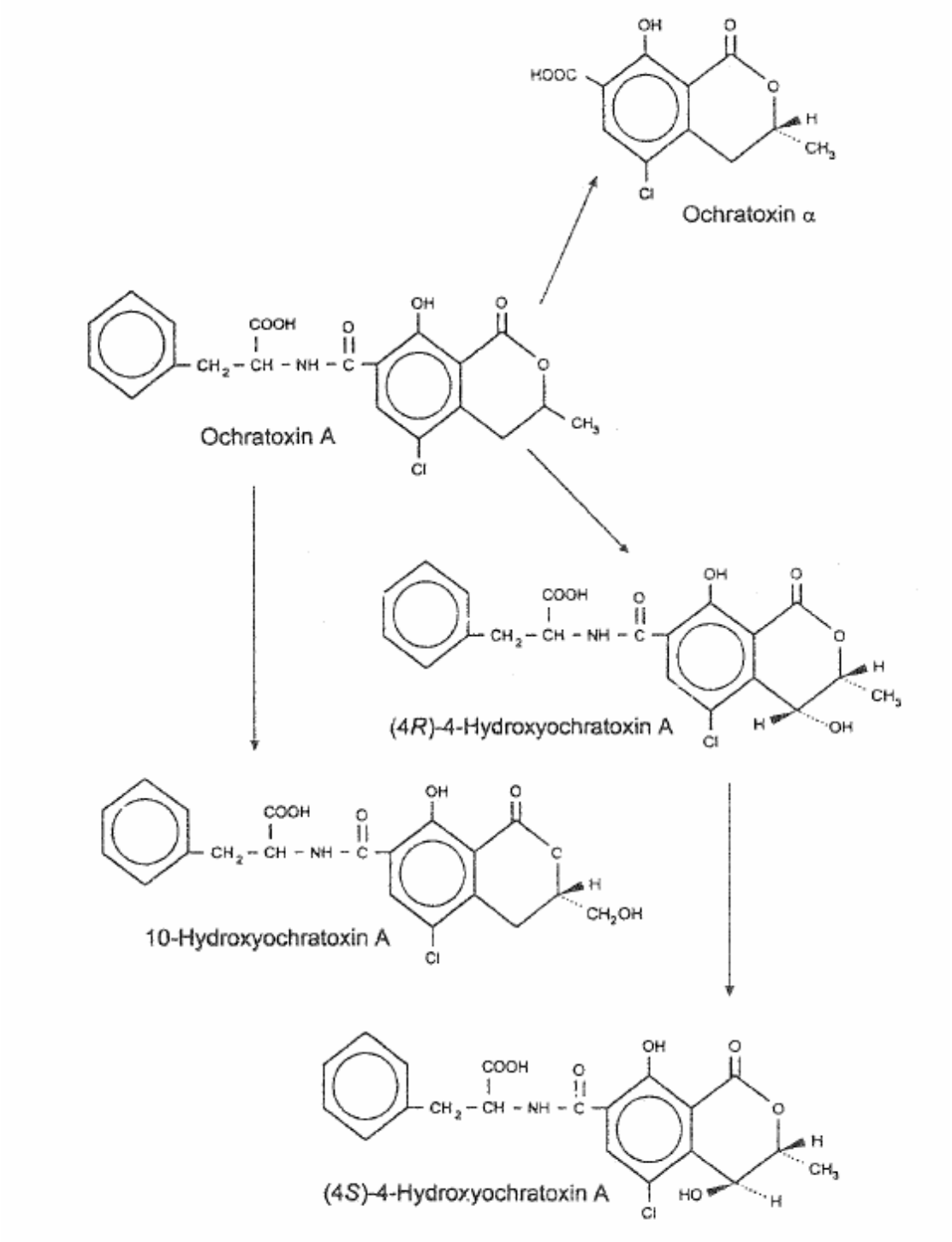

Fig 2.12 Metabolism of OTA (Neal, 1998).

OTA is slowly absorbed from the gastrointestinal tract. Distribution in a number of species is via blood, mainly to the kidneys, lower concentrations being

found in liver, muscle and fat. Transfer to milk has been demonstrated in rats, rabbits and humans, but little is transferred to the milk of ruminants owing to metabolism of OTA by the rumen microflora. OTA is only partly metabolized, most OTA is excreted unchanged. The major metabolite of OTA in all species examined is OTα. This and minor metabolites have been identified are all reported to be less toxic than OTA itself. OTA is excreted in urine and feces and the relative contribution of each of these excretory routes in different species is influenced by the extent of the enterohepatic recirculation of OTA and its binding to serum macromolecules. These factors are also important in the determination of the serum half-life of OTA, which varies widely among species. OTA has a long half-life in non-ruminant mammals, e.g. 24-39 h in mice, 55-120 h in rats, 72-120 h in pigs, 510 h in one macaque and 840 h in a human volunteer.

2.5.4 Mechanism of action

OTA is a structural analogue of L-β-Phe with a 7-dihydroisocoumarin chlorinated in position 5 (Van de Merwe et al., 1965). The chlorine atom seems to play a key role due to electronegativity because the dechloro derivative (OTB) is much less cytotoxic or not toxic at all (Roth et al., 1989). When introduced in the body, OTA binds strongly to plasma proteins and is metabolized into hydroxylated compounds, one of which, the (4R)-4-hydroxyochratoxin A being as cytotoxic and immunosuppressive as the parent compound in vivo and vitro (Creppy et al., 1983 a,c). OTA can be cleaved into phenylalanine (Phe) and OTα, the chlorinated dihydroisocoumarinic moiety found not to be toxic, but retaining some genotoxicity (Föllmann et al., 1995).

2.5.4.1 Inhibition of protein synthesis

OTA inhibits protein synthesis in vivo and in vitro at the elongation step by inhibiting the binding of Phe to its specific t-RNA (Bunge et al., 1978). This mechanism has been confirmed by the synthesis of analogues of OTA possessing other amino acids instead of Phe. They inhibit the reactions catalysed by the specific amnioacyl-tRNA synthetases appropriate to the amino acid replacing Phe in their structure (Creppy et al., 1983c).

In the meantime natural analogues have been isolated by Hadidane et al, 1992 indicating that in the case of natural contaminations, humans and animals are exposed to a mixture of toxins with consequent synergistic effects. It is well known that natural contamination by OTA-producing fungi is more potent in inducing porcine nephropathy than is the artificially OTA-contaminated feed for the same OTA levels (Mortensen et al., 1983).

Acute and chronic toxicity of OTA are related directly or indirectly to its property of inhibiting protein synthesis by competition with Phe in the reaction catalysed by phenylalanyl-tRNA synthetase (Heller and Röschenthaler, 1977). OTA is also able to inhibit all the reactions in which Phe, its structural analogue, is involved, such as those catalysed by Phe hydroxylase (Creppy et al., 1990). The same considerations apply to all those reactions involving the amino acid structural analogues of OTA such as tyrosine hydroxylase leading to DOPA for tyrosine-OTA which is cytotoxic (Creppy et al., 1983c).

These inhibitory properties of OTA, which explain almost all the toxic effects of OTA, include effects on certain enzymes involved in its metabolism, cytochrome P450 (P450), amino-pyrine demethylase and alanine hydroxylase levels are all

reduced by OTA at doses of about 1.5 mg/kg/15 days (Galtier et al., 1984). Phosphoenol pyruvate carboxykinase activity is also reduced in the kidney (Meisner et al., 1983).

2.5.4.2 Implication of oxidative pathways

Toxic effects of ochratoxins are also related at least partially to the oxidative processes, to the intracellular handling of calcium (Khan et al., 1989), to the inhibition of mitochondrial respiration and ATP production (Meisner and Chang, 1974). All these reactions result from the inhibition of protein synthesis. Rahimtula et al, 1988 have shown that OTA induces lipid peroxidation in the presence of microsomes and NADPH and traces of chelated Fe^{3+}. This lipid peroxidation is determined, in vitro, by malonaldehyde formation and, in vivo, by hexane exhalation. The first consequence of this harmful mechanism is the modification of membrane permeability and, consequently, cellular necrosis.

The production of free radicals is generally prevented by antioxidant and radical scavengers such as vitamin E, vitamin A, and superoxide dismutase (SOD) combined with catalase. The other oxidative pathways implicated in the metabolism of OTA are related to the cooxidation during prostaglandin (PG) synthesis. For the prevention of these reactions, inhibitors of PG synthesis and of cyclooxygenase could be used. Some of these inhibitors only partially block this pathway; others can block completely the whole system, for example piroxicam (Carty et al., 1980).

2.5.5 Toxicological studies

The toxicology of OTA has been already reviewed (Boorman, 1989), but some recent developments should be considered. In man OTA exhibits unusuals toxicokinetics, with a half-life in blood of 840 h (35 days) after oral ingestion (Schlatter et al., 1996). The delayed excretion of the toxin in man may be due to reabsorption during an enterohepatic circulation, due to reabsorption from the urine after tubular secretion, and due to extensive protein binding. Since the toxin is ingested with almost every meal, human may not be free of toxin for very long periods. The toxin has been considered by the International Agency for Research on Cancer to be possibly carcinogenic (group 2B) for humans (IARC, 1993), meaning that steady toxin exposure must be considered as a cause for serious concern. With respect to chemical carcinogens, not only dosage but, more importantly, the time-dosage profile (*c* X *t* product) has to be considered as relevant for tumor development. In this respect the ubiquitous presence of OTA is a subject of a toxicological debate presently (Petzinger and Ziegler, 2000).

Liver elimination of OTA is maintained by protein carriers that shuffle the toxin from its protein-bound form in blood into hepatocyte and subsequently secrete the toxin into bile. The uptake carrier has been identified (Kontaxi et al., 1996). But less is known about the mechanism involved in the release into bile. A carrier system is also involved in the uptake of OTA by proximal tubule cells, which secrete the toxin into urine (Tsuda et al., 1999). Such systems are biological entrance gates that determine the elimination toxicokinetics of OTA and therefore have a major impact on half-life times and selective organ exposure.

2.5.5.1 Acute and subacute effects

Species as well as sex and route of administration affect the toxicity of ochratoxin A. LD_{50} values range from 3.4 mg/kg (BW) for white leghorn chickens to 30.3 for male rats. Female rats are more sensitive. The intraperitoneal route of administration is more effective than the oral route (Krogh, 1987). The kidney is a target organ, as evidenced by functional and morphological changes, but other effects have been observed as well for example, liver and heart morphology abnormalities, blood clotting, myelotoxicity, intestinal fragility, reduced egg production, gastrointestinal/ renal and lymphoid tissue lesions. Moreover, cellular materials were condensed and membranes disappeared in the proximal convoluted tubules (Elling et al., 1985).

2.5.5.2 Carcinogenicity

Ochratoxin A is reasonably anticipated to be a human carcinogen based on sufficient evidence of carcinogenicity in experimental animals (Group 2B carcinogen). When administered by gavage, ochratoxin A substantially increased the incidence of uncommon tubular cell adenomas and of tubular cell carcinomas of the kidney in male and female rats. OTA also increased the incidence and multiplicity of fibroadenomas of the mammary gland in female rats (NTP 358, 1989). When ochratoxin A was administered in the diet, renal adenomas and carcinomas were observed in male mice, and some hepatocellular carcinomas were observed in female mice in one study. In another study, administration of ochratoxin A in the diet induced hepatomas and renal cell tumor in male mice. Other studies by dietary administration and studies by subcutaneous injection to mice and rats were considered inadequate in

term of the numbers of animals used and survival rates by an IARC Working Group. Based on these observations, the IARC Working Group considered the evidence for the carcinogenicity of ochratoxin A to be limited.

There are no adequate data available to evaluate the carcinogenicity of ochratoxin A in humans. Incidence of and mortality from urothelial urinary tract tumors have been correlated with the geographical distribution of Balkan endemic nephropathy in Bulgaria and Yugoslavia. A relatively high frequency of contamination of cereals and bread with ochratoxin A has been reported in an area of Yugoslavia where Balkan endemic nephropathy is present. No report of a direct association between ochratoxin A and human cancer is available (IARC V.10, 1976).

2.5.5.3 Mutagenicity and genotoxicity

Ochratoxin A is negative in conventional mutagenicity tests carried out according to standard protocol, i.e. Ames test and tests for gene mutations and chromosomal aberrations in mammalian cell cultures. However, using different test conditions and/or different endpoints, ochratoxin A is reported to be able to cause DNA-strand breaks *in vitro* and *in vivo*, micronuclei, unscheduled DNA synthesis, sister chromatid exchanges in vitro, gene mutations in bacterial cells (modified Ames test) and in NIH/3T3 cell line (Dörrenhaus and Föllmann, 1997).

2.5.5.4 Reproductive effects

Abnormalities involving limbs, neck, viscera and body size have been noted in chick embryos after the injection of ochratoxin A into the air sacs of fertile eggs at doses as low as 0.0005 mg/egg (Gilani et al., 1978). Teratogenic as well as other

developmental effects, for example fetal malformations to prenatal mortality, exencephaly and anomalies of the eyes, reduced prenatal survival, inhibited fetal growth, have resulted from treatment of pregnant mice, rats and hamsters with ochratoxin. Digits and tail were the most common defects (Hayes et al., 1974). The extent of gross malformations associated with ochratoxin A were dependent on the dietary protein level; eye, limb and tail malformations increased in the lowest dietary protein group, whereas skull and facial defects were noted in all groups (Singh and Hood, 1985).

2.5.5.5 Immunotoxicity

OTA can produce defects in the structure and/or function of elements comprising the immune system. Toxic effects resulting from very low concentrations of OTA in the ng/ml range affect the immune system. It appears that this system is by far the most sensitive among all other sensitive organs. OTA is clearly an immunosuppressive agent (Harvey et al., 1992). Concentrations as low as 5 ng OTA per kg BW suppressed immune responses in mice (Haubeck et al., 1981). Concerning the cellular immunity OTA leads to inhibition of immune responses transmitted by B- and T-lymphocytes. In relation to the humoral immunity OTA induces a regression of IgG-, IgA-, and IgM-immunoglobulins (Müller et al., 1995). To date there is no information regarding an OTA-induced cytokine release.

2.5.5.6 Metabolic effects

One of the first observed and most dramatic effects of ochratoxin A on metabolic systems was its *in vitro* and *in vivo* inhibition of protein synthesis in the

liver. This inhibition was prevented by administering phenylalanine concurrently with the toxin, which was evidence that the inhibition resulted from competition of ochratoxin with phenylalanine in the reaction catalyzed by phenylalanyl-t-RNA synthetase (Creppy et al., 1984). Ochratoxin A concentrations greater than $5x10^{-6}$ M inhibited RNA, DNA and protein synthesis at about the same degree in mouse L cells (Jeffery et al., 1984) Braunberg et al. (1988) found that 10^{-6} M ochratoxin A inhibited RNA, DNA and protein synthesis. At 25 μM, OTA inhibited the activity of several renal tubular enzymes (e.g., gamma-glutamyl transferase, leucine aminopeptidase and N-acetyl-β-D-gloucosaminidase), and also inhibited macromolecular synthesis and decreased renal tubule enzymes (Creppy et al., 1986).

Renal gluconeogenesis and the activity of cytosolic phosphoenolpyruvate carboxykinase (PEPCK) were reduced. The level of mRNA for this enzyme was also decreased but not its synthesis, indicating that the effect of the toxin was to change the mRNA abundance at the post-transcriptional level (Meisner and Polsinelli, 1986).

Another enzyme involved in carbohydrate metabolism, protein kinase, was studied. The activity of this enzyme, which initiates the cascading glycogen phosphorylase system, was inhibited by the lowest level of ochratoxin fed 0.5 mg/kg of diet (Warren and Hamilton, 1980). Activities of hepatic mixed function oxidase enzymes, including aminopyrene demethylase and aniline hydroxylase, and levels of cytochrome P-450 were decreased in rat administered ochratoxin A by intubation at a daily dose of 1.5 mg/kg (bw) for 15 days. Hepatic phase II biotransformation enzyme activities were unaffected (Galtier et al., 1984).

Several studies have been carried out by using in vitro systems to explore possible mechanisms responsible for the nephrotoxic action of ochratoxin A. A

number of studies have shown that ochratoxin A interferes with both the organic anion (e.g. para-aminohippurate or PAH) and the organic cation (e.g. tetraethylammonium acetate) (Berndt and Hayes, 1979). OTA transport in the kidney is mediated by the same renal organic anion transport systems (Friis et al., 1988). Since this is an active transport system requiring adenosine triphosphate (ATP) as the energy source, it is of interest that ochratoxin has also been demonstrated to reduce the cellular ATP contents of nephron segments in vitro at concentrations as low as 10^{-8} M.

In addition, ATP synthesis in mitochondria isolated from the renal cortex was significantly inhibited by 10^{-6} M OTA (Jung and Hitoshi, 1989). These findings suggest that both this action and competition by organic anions and OTA for a common carrier may be responsible for the inhibition of organic ion transport by the toxin.

Berndt et al. (1984) reported that calcium accumulation was stimulated by the addition of 10^{-5} M ochratoxin A to rat renal cortical slices. Finally, at a concentration of 125 μM, OTA greatly enhanced the rate of lipid peroxidation of rat liver microsomes as measured by malondialdehyde formation.

A more recent study involving an ultrastructural investigation of kidney in ochratoxin-treated pigs revealed a loss of membrane integrity of peroxisomes in the tubular epithelial cells along with enhanced levels of peroxisomal β-oxidation in the soluble kidney fraction (Elling et al., 1985), strengthening the possibility that oxidation-induced membrane damage is a factor in the pathogenesis of OTA toxicity. Investigating the mechanism of ochratoxin A-induced lipid peroxidation with reconstituted system of phospholipid vesicles, flavoprotein and iron ions. Omar et al.

(1990) reported evidence that the toxin stimulates the production of an iron-oxygen complex which initiates the peroxidation reaction.

2.5.5.7 OTA and Cytokines

Liver is a defense mechanism against bacteria and endotoxins after their intestinal absorption. The delivery of mycotoxins from the ingesta to the liver means that this organ is also an early protection barrier against a systemic toxin distribution throughout the entire body. Therefore, two factors were considered to be of importance for the acute toxin burden in the liver: the OTA elimination capacity of the liver and the acute response by cytokine effects.

Tumor necrosis factor α (TNFα) is among the early response cytokines, released following exposure of the liver to bacterial endotoxins, e.g., *E.coli*-lipopolysaccharides. Weidenbach et al. (2000) have recently demonstrated that this cytokine is also released in significant amounts from a blood-free perfused rat liver following OTA exposure. In addition, another pro-inflammatory cytokine, IL-6 was present in the perfusate. This cytokine release was additive to a prevailing TNFα release induced by *E.coli*. Therefore, OTA-related TNFα release in blood may provide an additional risk for sepsis patients. In contrast, other mycotoxins such as 3-acetoxy-deoxynivalenol (3-Ac-DON), xanthomegnin (XAN), citrinin (CIT), and viomellein (VIO) even suppressed OTA-induced TNFα release when present simultaneously in the perfusate (Figure 2.13)

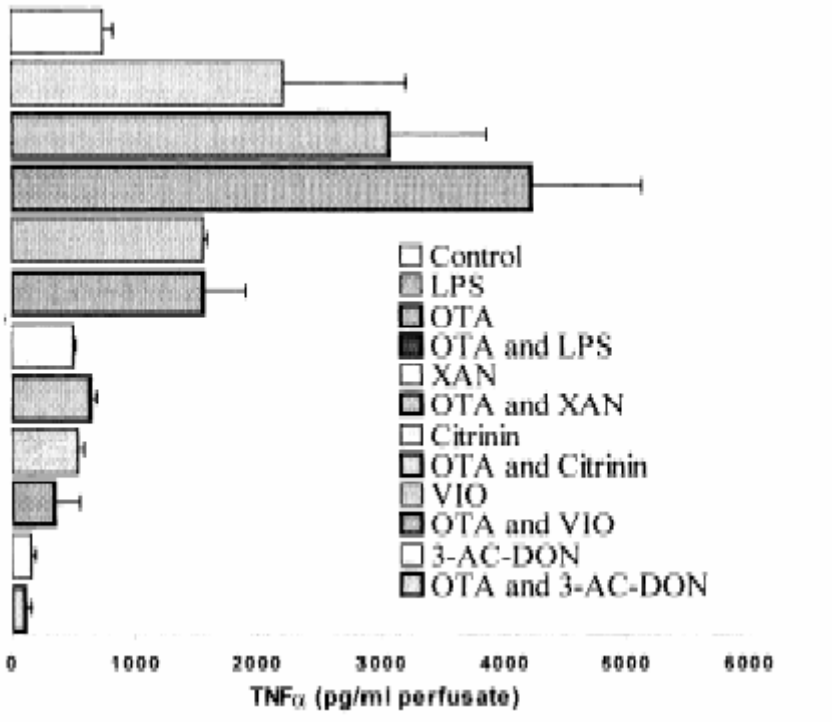

Fig 2.13 TNFα release from the isolated and perfused rat liver by LPS and mycotoxins.

Since the cytokine release was exclusively dependent upon extracellular calcium and was inhibited by the phosphodiesterase IV-inhibitor rolipram. Weidenbach et al. 2000 conclude that OTA represents a receptor-dependent trigger of a signal cascade leading eventually to TNFα secretion by liver cells (Kupffer cells and parenchymal cells). With respect to TNFα it is tempting to speculate that this cytokine may induce apoptosis in the liver, as has previously been reported in a kidney cell line (Schwerdt et al., 2000) and in various other cells (Seegers et al., 1994a,b).

2.5.5.8 Apoptosis

OTA induces apoptosis (programmed cell death) in a variety of cell types *in vivo* and *in vitro*. The mechanisms include caspase 3 activation, mitogen-activated protein kinase (MAPK) family, and c-jun amino-terminal kinase (JNK). The apoptosis is also mediated through cellular processes involved in the degradation of DNA. Finally, the metabolisms leading to cell death may be inhibited by various antioxidants. (Schwerdt, 1999)

2.5.6 Effect of processing

OTA is relatively stable and is only partially degraded under normal cooking or processing conditions. Milling has been reported to substantially reduce the concentration of OTA in white flour, but it has little effect on levels in whole meal flour. Milling is a physical process: the OTA removed from the grain in the production of white flour remains in bran and other fractions, some of which may be used in foods. OTA is relatively stable to heat: at 100°C, a 50% reduction in the concentration was achieved after 2.3 hour in wet wheat and 12 hour in dry wheat. The process involved in the manufacture of breakfast cereals and biscuits resulted in substantial reductions in ochratoxin content, but little or no reduction was found in the manufacture of egg noodles and pasta. Decaffeination of coffee reduces the ochratoxin concentration by about 90%. The reduction obtained by roasting coffee varies but may also be as much as 90%. OTA survives most food processing stages such as cooking, roasting, and fermenting to an appreciable degree and OTA can be detected in manufactured food products. The fate of ochratoxin A after process and cooking has been investigated (Table 2.14) with mixed results.

Table 2.14 Ochratoxin A: Fate during processing. (Pohland et al., 1992)

Commodity	Process	% Reduction
Barley (4 ng/g)	Ensiling	68
Cheddar cheese	Storage (48 h, 25°C)	41
Coffee beans	Roasting	97-100
Coffee beans	200°C, 10-20 min	0-12
Coffee, green	198-240°C	80-88
Coffee, ground	Brewing	100
Corn	Wet milling	4% in germ 51% in bits
Corn	Ammoniation (96h, 70°C, 5% NH_3)	95%
Faba beans	Cooking	16-20
Flour, bread	Baking (220°C, 25 min)	0
Flour, Biscuits	Baking (180°C, 5½ min)	62
Pig kidneys	Storage (-18°C, 7½ years)	0
Polished wheat	Cooking	6

The experimental results reflect the general thermal/hydrolytic stability of ochratoxin A, although it should be remembered that one of the problems encountered in developing analytical methods for ochratoxin A in grains was the instability (presumably enzymatic) of this compound after grinding of the grain during test sample preparation. In the processing of flours contaminated with ochratoxin A, it appears that some loss of toxin occurs, depending on the end product. The preparation

of blood sausages and puddings results in slight losses. Roasting of coffee beans results in anywhere from 12 to 100% destruction of ochratoxin A depending on the conditions used; brewing of coffee results in 100% loss of ochratoxin A. As was the case with aflatoxin, ammoniation is effective in removing ochratoxin A.

2.5.7 Interaction of ochratoxin A with other mycotoxins

There are most of the studies investigating possible interactions between ochratoxin A and other mycotoxins involved examination of the effets of these two toxins, acting separately and together, on biological system. As shown in table 2.15, the results depended on the biological system used, and ranged from antagonism to additive effects to synergism or enhanced effects. Also to be noted is the finding that whether or not an interaction is observed in a particular system is sometimes dependent on the particular parameter of measurement applied in that system.

Finally, other compounds besides mycotoxins are also reported to interact with ochratoxin A. Caffeine was reported to enhance the disturbance of fetus development induced by ochratoxin A (Tsubouchi, 1987). Biscoumacetate or phenylbutazone treatment of rats increased the toxicity of OTA when administered together with the toxin, possibly because of displacement of OTA from binding sites on plasma proteins, which was determined *in vitro* (Galtier et al., 1979)

Table 2.15 Interaction of ochratoxin A with other mycotoxins. (Pohland et al., 1992)

Mycotoxin	Test system/Measurment	Effect
Ochratoxin B	Rat renal tubular morphology	Greatly reduced (antagonistic)
Ochratoxin B	Mouse liver-protein synthesis	No effect or additive
Citrinin	Hepatoma cells-RNA, DNA, protein synthesis	Synergistic
Citrinin	Renal cortical cells-organic ion transport	Additive or slightly synergistic
Citrinin	White leghorn pullets-kidney function	Reduced citrinin effect
Citrinin	Layer chick-renal ultrastructure	Additive
Citrinin	Mouse-hepatorenal carcinogenesis	Synergistic (only for renal effect)
Citrinin	Chick embryo-morphology	Additive
Citrinin	Broiler chicks-growth depression, water consumption increase	Reduced (antagonistic)
Citrinin	Fetal rat-malformation	Synergistic
Citrinin	Rat-renal Na^+-K^+ and Mg ATPase	Synergistic (only Na^+-K^+ ATPase)
Citrinin	Mouse-lethal effect	Synergistic
Citrinin	Guinea pig-lethal effect	Synergistic (female), Additive (male)

Table 2.15 Continued.

Mycotoxin	Test system/Measurment	Effect
T-2 toxin	Broiler chickens-body weight, serum protein LDH triglyceride, GGt and Ca^{++}	Antagonistic for Ca^{++} and GGt, Synergistic for triglyceride
Aflatoxin	Poultry-fatty livers and growth weight	Reduced aflatoxin effect for fatty liver, Synergistic for growth weight and nephropathy
Aflatoxin B_1	Swine-renal interstitial fibrosis	Mild antagonism
DON	Broiler chick-general toxicity	Reduced (antagonistic)
Zearalenone	Fetal mouse-teratogenesis	Reduced (antagonistic)
Penicillic acid	Mouse-renal tubular morphology	Synergistic
Penicillic acid	Mouse-lethal effect and RNA synthesis	Synergistic

2.5.8 Methods of ochratoxin A decontamination

Decontamination/ detoxification procedures are useful in order to recuperate mycotoxin contaminated commodities. Several strategies are available for the detoxification of mycotoxins. These can be classified as physical, chemical and (micro) biological approaches.

The physical methods are focused on the removal of mycotoxins by different adsorbents added to mycotoxin-contaminated diets (Ramos et al., 1996a) with the hope of being effective in the gastro-intestinal tract more in a prophylactic rather than in a therapeutic manner. At present, however, the utilization of mycotoxin-binding adsorbents is the most applied way of protecting animals against the harmful effects of decontaminated feed.

2.5.8.1 Physical treatment

2.5.8.1.1 Activated charcoal

Activated charcoal which is formed by pyrolysis of organic materials is a very porous non-soluble powder with a high surface to mass ratio (500–3500 m^2/g). Since the 19th century it has been used as an antidote against poisoning. Therefore, it might also inactivate mycotoxins. In aqueous solution, it can adsorb most of the mycotoxins efficiently (Table 2.16) whereas different activated charcoals have less or even no effects against mycotoxicosis (Table 2.17). This might be due to the fact that activated charcoal is a relatively unspecific adsorbent and, hence, essential nutrients are also adsorbed particularly if their concentrations in the feed are much higher compared to those of the mycotoxin. In other trials with goats, however, it was shown that high

doses of activated charcoal are beneficial in an acute poisoning situation concerning the intake of high amounts of aflatoxins (Hatch et al., 1982).

2.5.8.1.2 Aluminosilicates (Zeolites, HSCAS, clays)

Most studies related to the alleviation of mycotoxicosis by the use of adsorbents are focused on aluminosilicates, mainly zeolites and hydrated sodium calcium aluminosilicates (HSCAS), and aluminosilicate-containing clays, all consisting of aluminates, silicates and some interchangeable ions, mainly alkali metal and alkaline earth metal ions (Barrer, 1989). Clay minerals are primarily layered silicates with the common chemical formula $[Si_2O_5{}^{2-}]_{xy}$, e.g. kaolin $Al_4(OH)_8Si_2O_5$. Zeolites are composed of tetrahedrons of SiO_4 and AlO_4 as the two fundamental building blocks with the metal atom at the center of each tetrahedron. The common chemical formula is $[AlSi_3O_8{}^{-}]_{xyz}$, e.g. orthoklas $KAlSi_3O_8$, zeolite A $\{Na_{12}[Al_{12}Si_{12}O_{48}]\cdot 27\ H_2O\}_8$. While the SiO_4-unit is electrically neutral, the AlO_4-unit carries one negative charge which has to be compensated by positive charges, usually sodium ions as in zeolite A. Zeolites are similar to molecular sieves as well as to ion exchange resins and are suitable for the distinction of different molecules by size, shape, and charge. HSCAS contain calcium ions and protons which are exchanged against the naturally occuring sodium ions. They are a type of montmorillonite belonging to phyllosilicates which are composed of layers of aluminium and silicon connected in a 1:1 or 2:1 arrangement.

Regarding the applicability of aluminosilicates for the binding of mycotoxins, it can be concluded that they are very effective in preventing aflatoxicosis, but their efficacy against zearalenone, ochratoxin, and trichothecenes is limited. In addition to

the narrow binding range concerning different mycotoxins, aluminosilicates have the disadvantage of showing high inclusion rates for vitamins and minerals.

Table 2.16 *In vitro* adsorption of ochratoxin by different adsorbents (Huwig et al., 2001).

Adsorbent	**Mycotoxin**	**Adsorption capacity (mg/g)**
Activated charcoal	och	100
Bentonite	och	1.5-9.0
HSCAS	och	0-2.2
Yeast[a]	och	1.2-8.6
Modified yeast cell walls extract	afl/ och/ zea/ tri	0.2-1.9
Cholestyramine	och	9.6

afl, aflatoxin; och, ochratoxin A; zea, zearalenone; tri, trichothecenes

[a] 40% sterilized yeast, 60% fermentation residua of beer production.

Table 2.17 *In vivo* adsorption of mycotoxins by different adsorbents (Huwig et al., 2001).

Adsorbent	Concentration (%)	Mycotoxin	Effects observed
Activated charcoal	10.0	och	Significant reduction of the och concentration in blood, bile, tissues of pigs.
HSCAS	0.5	afl/och	Growth inhibitory effects on chickens diminished by 65%, no effect against toxicity of och, little effect against toxicity of combined toxins.
HSCAS	1.0	och	No significant effect (pigs).
Bentonite	1.0/10.0	och	No significant effect (pigs).
Yeast[a]	5.0	och	No reduction of the och concentration in blood, bile, tissues of pigs.
Cholestyramine	1.0	och	No reduction of the och concentration in blood, bile, tissues of pigs

afl, aflatoxin; och, ochratoxin A; zea, zearalenone; tri, trichothecenes

[a] 40% sterilized yeast, 60% fermentation residua of beer production.

2.5.8.1.3 Polymers

Cholestyramine is an anion exchange resin which is used for the binding of bile acids in the gastro-intestinal tract and for the reduction of low density lipoproteins and cholesterol. The in vitro binding capacity of this resin for ochratoxin A and zearalenone was 9.6 mg/g and more than 0.3 mg/g (Ramos et al., 1996b), respectively, but in vivo, cholestyramine had only a very small effect on the reduction of the ochratoxin concentration in blood, bile, and tissues. Another adsorbent is crospovidone (polyvinylpyrrolidone), a highly polar amphoteric polymer the in vitro adsorbance of which was measured as 0.3 mg/g for zearalenone by Ramos et al. (1996b). Up to now, this polymer has not been tested in vivo. An improved cryogel of cross-linked polyvinylpyrrolidone recently showed increased values up to 2.1 mg/g (Alegakis et al., 1999).

2.5.8.2 Chemical treatment

Chemically, some mycotoxins can be destroyed with calcium hydroxide monoethylamine, ozone (McKenzie et al., 1997) or ammonia (Park, 1993). Particularly the ammoniation is an approved procedure for the detoxication of aflatoxin-contaminated feed in some U.S. states as well as in Senegal, France, and the UK. The average ammoniation costs vary between 5 and 20% of the value of the commodity (Coker, 1998). Main drawbacks of this kind of chemical detoxication are the ineffectiveness against other mycotoxins and the possible deterioration of the animal health by excessive residual ammonia in the feed.

As for Aflatoxin others chemical treatments have been proposed:

- Treatment with hypochlorite

- Treatment with Ozone
- Alkaline hydrogen peroxide treatment

2.5.8.2.1 Treatment with Phenylalanine and piroxicam

Phenylalanine (Phe) and piroxicam favour the absorption of OTA from the stomach but seem to prevent it from being distributed to all organs. In using these compounds the blood-OTA concentration was effectively higher but the intestinal absorption decreased. Urinary elimination was enhanced by 70% and 110% for Phe and piroxicam, respectively, within the first 24 h. In normal conditions and after dosing rats for 2-6 weeks with 289 µg/kg/48 h of OTA, the blood contained 3.3-3.6 µg/ml while the urine contained 0.13-0.24 µg/ml (Baudrimont et al., 1995a).

Phe influences the toxicokinetics of OTA. It increases gastrointestinal absorption, intestinal transit, urinary and intestinal elimination. The half-life time is reduced, and the acute toxicity is largely prevented (Meisner et al., 1983).

It should be remembered that Phe has no effect at all on the genotoxicity of OTA (Kane et al., 1986a), indicating that the reactive compounds responsible for this genotoxicity are not structurally related to Phe. This includes Tyr-OTA, OTα and perhaps conjugates of OTA as candidates for these harmful chronic effects.

Phe is very efficient on protein synthesis inhibition induced by OTA (Creppy et al., 1983c).

2.5.8.2.2 A19 as detoxicating agent (Creppy et al., 1995)

A19 is Aspartame, i.e. the methyl ester of aspartyl Phe. It is thus a structural analogue of both Phe and OTA. A19 was completely cleaved into aspartate, Phe and methanol within a few hours.

A19 is absorbed from stomach and intestine into the blood both under the unchanged (10-12% of the given dose) and cleaved form (aspartate and Phe mainly). The cleavage is time dependent. That gives rise to a pool of Phe which is much more slowly cleared from the body than the one provided by food proteins. The so provided Phe plays most probably a role as detoxicating agent (prevention of protein synthesis inhibition induced by OTA etc.). The unchanged A19 (10-12% of the given dose) which is much more than OTA concentrations in the body in case of natural contamination prevents OTA binding to plasma proteins, enhances OTA elimination, enhances OTA metabolism, especially to less toxic and genotoxic metabolites. The results confirmed decreasing OTA distribution in organs such as kidney, brain, liver and testicles and finally the prevention of OTA-induced nephrotoxicity and genotoxicity.

2.5.8.3 Biological treatment

Biological methods are not yet used in practice though the number of corresponding patents increases continously (Duvick and Rood, 2000). These methods include fermentation procedures with microorganisms. One example is the conversion of aflatoxin B_1 (particularly by *Flavobacterium auranticum*) to harmless degradation products. The conversions, however, are generally slow and incomplete (Sweeney and Dobson, 1998).

2.5.8.3.1 Yeast and products from yeast

Besides its excellent nutritional value, yeast or yeast cell walls can also be used as adsorbents for mycotoxins. The in vitro adsorption of ochratoxin by yeast (consisting of 40% sterilized yeast and 60% fermentation residual of yeasts used for beer production) is dependent on the pH being at maximum in acidic solutions (at pH 3: 8.6 mg/g, at pH 8: 1.2 mg/g). However, in trials with pigs employing a feed supplement of 5% of yeast, only a slight reduction of the ochratoxin A concentration in blood plasma, bile, and tissues was achieved. By the use only of yeast cell walls instead of whole cells, the adsorption of mycotoxins can be enhanced. The cell walls harboring polysaccharides (glucan, mannan), proteins, and lipids exhibit numerous different and easy accessible adsorption centers including different adsorption mechanisms, e.g. hydrogen bonding, ionic, or hydrophobic interaction. Therefore, it was possible to bind 2.7 mg zearalenone per gram of cell walls. The binding was rapid and reached equilibrium after only 10 min, which is superior to commercial available clay-based toxin binders (Völkl and Karlovsky, 1998).

Bejaoui and co-worker (2004) demonstrated that heat and acid treated *S. cerevisiae* LALVIN Rhône 2056 enhanced significantly OTA removal from liquid media. Polysaccharides and peptidoglycans are both expected to be affected by heat and acid treatments. Heating may cause denaturation of proteins or formation of Maillard reaction products. Acidic conditions could affect polysaccharides by releasing monomers, further fragmented into aldehydes after the breaking down of glycosidic linkages. These released products could offer more adsorption sites than viable cells and may increase surfaces for OTA binding. Moreover, the decrease in wall thickness of peptidoglycan and/or the increase in its pore size under heat and acid

treatments could probably make available other sites from yeast cells for OTA adsorption. The acidity could probably improve the OTA adsorption mechanism. In conclusion, Ionic properties of the OTA molecule, yeast membrane state and biomass concentration were the major factors affecting the OTA adsorption phenomenon.

In another context, it was shown that yeast killer toxins were adsorbed by the polysaccharides and not by the proteins or fatty acids of yeast cell walls (Radler and Schmitt, 1987) and that this adsorption was not unspecific because cellulose and glycogen were not able to bind killer toxins.

2.5.8.3.2 Conidia of black *Aspergilli*

Bejaoui et al. (2006) reported that conidia of black *Aspergillus* isolate representing the species *Aspergillus niger*, *Aspergillus carbonarius*, and *Aspergillus japonicus* behave as the biological adsorbent. They described the adsorption mechanism as this following, adsorption phenomenon of OTA on conidia of black *aspergilli* could be related to hydrophobic interactions. The global positive charge of OTA molecule in acid media could also interact with negatively charged molecules found on fungal conidia. This interaction is likely to be nonspecific, as many conidia are able to bind nonspecifically or without requiring chemical compounds. After OTA adsorption by conidia of black *aspergilli*, OTA degradation took place by well-developed mycelium after 14 h for *A. japonicus* and 30 h for both *A. niger* and *A. carbonarius*. Thus, OTα, an OTA degradation product, also observed in different previous studies, increased while OTA disappeared from the grape juice. So, two stages were involved in OTA detoxification by *Aspergillus* section Nigri isolates: adsorption and then degradation. Commercially, the only stage very interesting is

adsorption because it is fast, avoiding long time contact between fungi and grape juice. So, potentially commercial OTA detoxification by using conidia of black *aspergilli* must be completed before the mycelium is well developed and degradation starts. Indeed, production of a degradation product like OTα that is even reported as less toxic would still be harmful for consumer health. Among three *Aspergillus* section Nigri isolates tested, conidia of *A. carbonarius* were in many cases the most efficient in adsorbing OTA, regardless of the media used and the OTA concentration tested. In fact, conidia of *A. carbonarius* had the largest diameter (7-10 µm) compared to conidia of *A. niger* (3.5-4 µm) and those of *A. japonicus* (5 µm) and so presented a higher surface to bind OTA.

2.5.8.3.3 Control by microorganism or their enzyme

Several reports describe OTA degrading activities of the microbial flora of the mammalian gastrointestinal tract including rumen microbes of cow and sheep (Galtier and Alvinerie, 1976; Hult et al., 1976; Park et al. 1988) and microbes living mainly in the caecum and large intestine of rats (Madhyastaha et al., 1992). The human intestinal microflora can also partially degrade OTA (Akiyama et al., 1997). The species responsible for OTA detoxification have not yet been identified, although mainly protozoa were suggested to take part in the biotransformation process in ruminants (Kiessling et al., 1984). In addition, *Butyrivibrio fibrisolvens*, a rumen bacterium, was also reported to detoxify OTA to some extent (Westlake et al., 1987). Degradation of OTA was observed in milk due to the action of *Lactobacillus, Streptococcus* and *Bifidobacterium* spp. (Skrinjar et al., 1996), while two other bacteria, *Acinetobacter calcoaceticus* (Hwang and Draughon, 1994) and

Phenylobacterium immobile (Wegst and Lingens, 1983) could also convert OTA to the much less toxic ochratoxin α in liquid cultures. Furthermore, recent reports describe the OTA degrading activities of some *Aspergillus* and *Pleurotus* isolates and/or their enzyme (Varga et al., 2000b). Carboxypeptidase was earlier found to be able to convert OTA to ochratoxin α (Deberghes et al., 1995).

2.5.9 Regulatory concentration limits for ochratoxin A in food products

Several countries established concentration limits for OTA in different food products. These limits are presented in Table 2.18.

Table 2.18 Concentration limits for OTA in food products.

Food product	Concentration limit (µg/kg)	Country	References
Cereals, non-processed	5	EU	Byrne, 2002
Cereals, processed	3	EU	Byrne, 2002
Cereals	20-50	Brazil, Israel	FAO, 1997
Cereals	2	Switzerland	FAO, 1997
Dried vine fruits	10	EU	Byrne,2002
Milk products	0.5*	Cyprus	FAO, 1997
Raw coffee beans	20	Greece	FAO, 1997
Pig kidneys	10	Denmark	FAO, 1997

* as sum of mycotoxins

2.6 Mycotoxin determination method

Figure 2.14 shows the flow diagram for mycotoxin analysis.

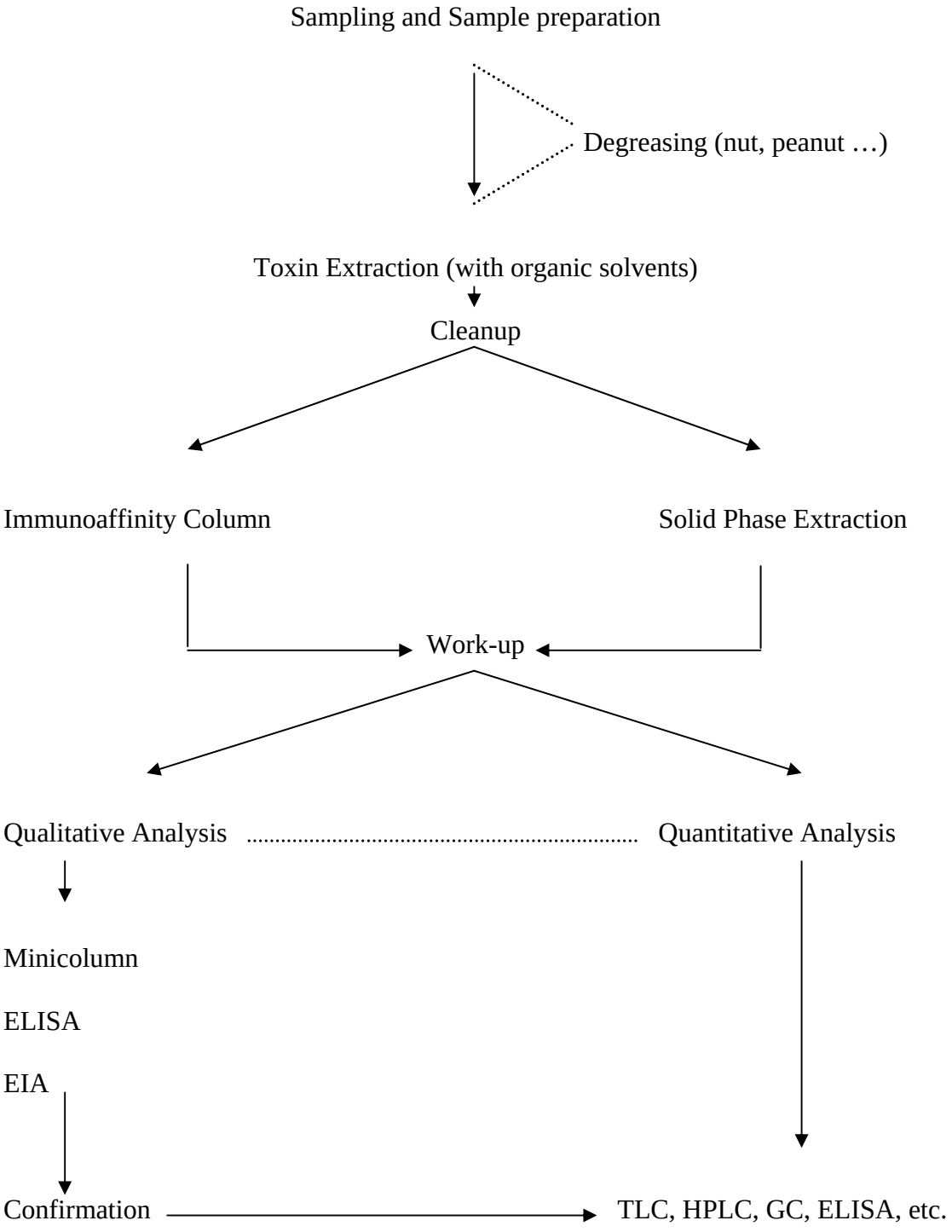

Fig. 2.14 Flow diagram for mycotoxin analysis.

2.6.1 Sampling

The first problem encountered with mycotoxin analysis is the selection of a representative sample. This problem is most severe with grain, nut, and oilseed commodities.

Techniques for sample preparation have also been published. In the case of seeds and nuts, these procedures involve reduction in particle size by grinding or milling for efficient extraction, along with good comminuting and mixing of the entire sample to obtain a representative portion for analysis. Stoloff (1972) recommends taking a large number of randomly scattered subsamples that have been finely ground and well mixed as the best approach to obtaining representative samples. The Official Methods of Analysis of the AOAC (1980) recommends that the entire laboratory lot sample be ground and thoroughly mixed, and that the subsamples be taken from this. In the case of free flowing powders, such as flours, and in the case of liquids or pastes, further size reduction may not be needed, although thorough mixing before removing the subsamples is still necessary. The equipment used for size reduction and mixing of samples is also discussed in AOAC's Official Methods of Analysis (1980). After thorough mixing, the subsamples should be taken with the same effort to be representative as was applied to taking the lot sample. Whenever possible, the sample should be subdivided by a random dividing procedure, such as riffling or similar means, until the desired sample size is achieved. When this type of subdivision is not possible, a composite number of small randomly taken portions should be used. In the case of liquids, any particulate matter should be resuspended before the samples are taken.

Sample size is an important consideration in obtaining representative samples. The uneven distribution of mycotoxins in a commodity makes it desirable to test an entire batch or lot of suspect material, but this course is impractical. Usually, lot samples are limited to sizes of 1 to 5 kg. From this sample the subsample is taken.

The size of subsamples also varies, depending upon the method of analysis, and has ranged from 20 to 100 g. A subsample size of 50 g is used by most methods and appears to be the best size to obtain both solvent economy and a representative sample.

2.6.2 Extraction

The extraction methods for aflatoxins are based on the solubility of these toxins in organic solvents, mainly chloroform, methanol, acetone, benzene, and acetonitrile. For more complex matrices, the addition of diatomaceous earth or citric acid is required. From matrices of vegetable origin, water is usually added in the extraction step, since it facilitates solvent penetration into substrates, improving the percentage of extraction of the toxin.

2.6.2.1 Extraction with Chloroform

A chloroform-water mixture is the extraction solvent for the contaminants branch (CB) method, proposed by Eppley (1974) The CB method has been adopted by the Association of Official Analytical Chemists as the official method for the determination of aflatoxins in groundnuts and their by-product.

Extraction with a mixture of chloroform-water is also used in the ECC method in feedstuffs, based on the Paulsch et al. (1988). method, and has been successfully

adopted for various food matrices, such as rice, spices, grains, and dried fruit sample: 50 g of finely ground sample are mixed with 25 g of diatomaceous earth, moistened with 25 ml of water, and thoroughly shaken to obtain a homogeneous blend. Two hundred and fifty ml of chloroform are added and the whole shaken vigorously for 30 min on a vibrating shaker. Fifty ml of chloroform extract, recovered by filtering through a folded filter paper, is used for subsequent purification and assay.

The diatomaceous earth retains various substances, such as pigments, by adsorption, which simplifies subsequent purification and at the same time facilitates filtration by preventing the filter from clogging.

2.6.2.2 Extraction with Methanol

A methanol /hexane mixture is used in the best food (BF) method to extract the toxin from the substrate: 100 g of sample is added to 500 ml of a methanol-water (55:45) mixture and 200 ml of hexane. After 1 min of vigorous shaking, the resulting solution is centrifuged for 5 min at 2000 rpm. Twenty-five ml of the methanol phase is used for the TLC assay. The method has been adopted by the AOAC Official Methods of Analysis in peanut products.

Methanol-water is the extraction medium of the method recently tested for validation by the European Commission, for the determination of aflatoxins at the European regulatory limits for dried figs, pistachios, peanut butter, and paprika: 50 g of the test portion are extracted with methanol-water (80:20) for dried figs and paprika, and methanol-water (80:20) plus 100 ml of hexane for peanut butter and pistachio. After filtration, the filtrate is added to phosphate buffer saline (PBS) for the purification step.

2.6.2.3 Extraction with Acetone

A mixture of acetone/water is used in the Roch et al. (1995). method for aflatoxins in groundnut cake: 1 kg of the sample is grounded to a fine powder and mixed using a Hobert Vertical Cutter Mill (VCM 25). Sixty gram of cake is extracted with 600 ml of acetone: water (85:15). The mixture is carefully shaken and filtered through a folded paper; 5 ml of the acetone extract is used for the subsequent purification assay.

2.6.2.4 Extraction with Acetonitrile

Acetonitrile-water is used in the Patey et al. (1990) method for the determination of aflatoxins in peanut butter: 10 g of peanut butter and 30 ml acetonitrile/water mixture are shaken. Forty-five ml of water is added, and the mixture is shaken for an additional 30 min. After centrifugation at 4000 rpm at 30°C, the supernatant is filtered, added to PBS, and gently mixed for 15 s.

2.6.3 Cleanup

The cleanup step is generally based on two different approaches: (a) the use of solid-phase extraction (SPE) cartridges, (b) the use of immunoaffinity (IA) columns.

2.6.3.1 SPE cartridges

Commonly silica, Florisil, or C_{18} cartridges or a combination of these are used. The major advantages of this methodology are the elimination of the column chromatography cleanup step, the possibility of regenerating the cartridge for further analyses, and the low cost. The disadvantages are associated with the potentially low

reproducibility of different batches of columns and/or low repeatability within a single batch.

2.6.3.2 IA columns

The use of antibody-based immunoaffinity columns has recently led to a considerable increase in the reliability of results, due to the high selectivity of this technique. Other advantages include the reduced the time of analysis, and the possibility of analyzing more than one sample simultaneously. Basically, the extract is purified into an immunoaffinity column containing antibodies specific to aflatoxins. The column is previously conditioned by applying 10 ml of PBS at a speed of 2-3 ml/min (gravity). The column is washed with water to remove impurities from the immunoaffinity column. Aflatoxins are separated from the antibody by passing methanol through the column. The resulting methanol solution can be injected into HPLC. An immunoaffinity column in the cleanup step is used in the method tested at the European Commission. More precision between samples results can be obtained using a workstation in which sample preparation, derivatization, and HPLC injection is automated.

2.6.4 HPLC Analysis

To date, two main principles of liquid chromatography have been used: adsorption chromatography and liquid partition chromatography. In consideration of the wide development of reversed-phase column and the consequent increasing applicability to various food matrices, adsorption chromatography has been almost entirely abandoned. Basically, in reversed-phase-partition chromatography, the

mobile phase is more polar than the stationary phase, and combinations of solvents such as methanol, acetonitrile, water, and, rather infrequently, acetone are currently used as mobile phases. As for the stationary phase, the use of silica as support with chemically bonded chemical groups such as octadecyl or octyl is quite common. The residual free sylanolic groups are saturated with C-2 or other groups in order to increase the percentage of carbon loading. The main aim is to achieve a reduction of tailing peaks with a more symmetrical shape. Such columns are labeled as ODS1, OSD2, OSD3, or fully endcapped.

As regards the aflatoxin detection, UV spectrophotometry has been definitively abandoned, because of the lack of selectivity and the very poor analytical response, resulting in a very high detection limit. Since aflatoxins show fluorescence under UV light spectrofluorimetric detection has been adopted in the last years.

The operating conditions range from an excitation wavelength of 360-365 nm and an emission wavelength 425-435 nm. The intensity of aflatoxin fluorescence depends strongly on the injected solvent, with a higher response if the sample is injected in the mobile phase (usually a ternary mixture of methanol/ acetonitrile/ water) and a lower one if injected in the methanol or acetonitrile only.

Furthermore, since aflatoxins B_1, G_1, and M_1 do not give a high response, it is necessary to derivatize them via pre- or postcolumn derivatization, in order to enhance their fluorescence.

2.6.4.1 Trifluoroacetic acid (TFA) precolumn derivatization

This reaction results in an aflatoxin hemiacetal formation (aflatoxins B_{2a}, G_{2a}, and M_{2a}) by the addition of a water molecule to the double bond of the furanic ring.

2.6.4.2 Postcolumn derivatization with iodine

The procedure has been developed by Tuinstra and Haasnoot (1983); a freshly prepared aqueous saturated solution of iodine (2 g in 400 ml of bidistilled water) is pumped by an auxiliary HPLC pump simultaneously with the mobile phase (commonly water/methanol/acetonitrile). The iodine solution must be kept away from UV light. The reaction is carried out in Teflon tubing (0.5 mm x 3000 mm) thermostated at 60°C. A silicone oil bath is preferred to a water bath for a more stable temperature. The flow rates of the mobile phase and the derivatizing agent are generally 0.8ml/min. and 0.7 ml/min, respectively.

2.6.4.3 Postcolumn derivatization with pyridinium bromide perbromide (PBPB)

By this method the brominating agent enhances the fluorescents signal of aflatoxins B_1 and G_1, increasing the sensitivity of the analytical method. The solution of PBPB (0.05 mg/ml in HPLC-grade water) is pumped at a flow rate of 0.3 ml/min, and the flow rate of the mobile phase is generally set at 1.0 ml/min.

2.6.4.4 Postcolumn derivatization with electrochemical-generated bromide (Kroba II)

Recntly the fluorescence signal enhancement of aflatoxins was obtained by an online derivatization, with electrochemical-generated bromine. The reagent is produced in a postcolumn electrochemical cell from the bromide present in the mobile phase. The reagent concentration is controlled by the generating current. For electrochemical production of bromine, a Kobra cell is used. Reaction coils, providing

reaction times of 4, 8, and 24 s at a flow rate of 0.5 ml/min, are used. The current is delivered by a variable DC supply with 100 kΩ of resistance in series with the Kobra cell.

2.6.4.5 Liquid chromatography-MS:

Mc Fadden and Schueler performed the analysis of aflatoxins B_1, B_2, G_1, and G_2 by ion reconstruction at m/z 313, 315, 329, and 331 for the four aflatoxins, respectively, by using a moving-belt interface. Tiebach et al. (1985) used a direct liquid induction (DLI) interface with a reversed-phase microbore LC system, and obtained positive-ion chemical ionization (PCI) and negative-ion chemical ionization (NCI) spectra for aflatoxin M_1 and reconstructed ion chromatograms for aflatoxins B_1, B_2, and M_1. Tateo et al. (2004) used an MS/DI system for confirmatory analysis of aflatoxins. A liquid chromatography/mass spectrometry interfacing method was reported by Cappiello et al. (1995). The Chromatographic separation was performed with a reversed-phase packed capillary column coupled with a modified particle beam interface capable of handling microliter-per-minute flow rates.

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CHAPTER III

ISOLATION, CHARACTERIZATION AND OCHRATOXIN A PRODUCTION ABILITY OF TOXIGENIC FUNGI FROM FRENCH GRAPE

3.1 Abstract

This work was realized in collaboration with Hend Bejaoui in the framework of her Ph'D study in 2004. A survey of toxigenic fungi from French grapes was conducted during year 2003 in order to study about their ochratoxin A (OTA) production ability. Ten bunches of each eight grape varieties were collected at each growth stages from 10 vineyards in 4 winemaking regions in France. All were analyzed and results indicated that the predominant microflora (80% ± 4.6) was *Aspergillus* genus represented by Section Nigri (99%) when compared to *Penicillium* (20% ± 4.6). Approximately 32.5% (±σ = 1.26) of the fungal isolates were OTA producers and 93% (±σ = 2.65) belonging to black aspergilli. The ochratoxigenic potential of the isolates and their occurrence on grapes revealed that *Aspergillus carbonarius* was the main OTA producer (up to 37.5 µg g^{-1}). In addition, grapes which were collected at harvest time had the maximal fungal population and this was the critical period for OTA contamination. Finally, grapes from Languedoc-Roussillon region were most infested with ochratoxigenic fungi than grapes from other region.

3.2 Introduction

Ochratoxin A is one of the group of mycotoxins produced as secondary metabolites by several fungi of the *Aspergillus* or *Penicillium* families and are weak organic acids consisting of a derivative of an isocoumarin. OTA comprise the first major group of mycotoxins indentified after the discovery of the aflatoxins. OTA has been concerned from both scientific and food committee as the secondary severe mycotoxins next from aflatoxins because it shows nephrotoxic (Krogh et al., 1974; Mortensen et al., 1983), carcinogenic (Boorman, 1989) and teratogenic (Arora and Fröelén, 1981) properties in animals. However, there is inadequate available evidence on the genetic and related effects of ochratoxin A in humans. Therefore, IARC classified OTA into Group 2B carcinogen (possibly carcinogenic to humans).

Thus Ochratoxin A is a toxic and potentially carcinogenic fungal toxin found in a variety of food commodities. There are several reports indicated that many food and feedstuffs were contaminated by OTA. It is found mainly in cereal and cereal products. Besides cereals and cereal products, ochratoxin A is also found in a range of other food commodities, including grain and grain products from rye, wheat, barley, oats, maize, buckwheat and millet (Wolff, 2000), flour (Kuiper-Goodman and Scott, 1989) some infant cereal foods from soy products, soybean (Krogh, 1987), bread and rolls (Cholmakov-Bodechtel et al., 2000), coffee (Studer-Rohr et al., 1995), beer (Jørgensen, 1998), cocoa, pulses, spices, dried fruits, pig kidney and other meat and meat products of non-ruminant animals exposed to feedstuffs contaminated with this mycotoxin. Additionally, OTA also contaminates wines and grape juices (Scott and Kanhere, 1995) and its derived products such as dried vine fruit (MacDonald et al., 1999). The frequency of the occurrence of the different species of ochratoxin A-

producing fungi differs according to the geographical regions and in the commodities affected.

France is one of the leading wine-producing countries in Europe and in the world. They produce 5,199,930 metric ton in year 2002 (FAO, 2002). France now produces the most wine by value in the world (although Italy rivals it by volume and Spain has more land under cultivation for wine grapes). France is also the top wine consumer world-wide, with an annual consumption per capita of 58 liters. Bordeaux wine, Bourgogne wine and Champagne are important agricultural and export products. Unfortunately, provisional estimation of the Codex Alimentarius Commission, based on limited European data, suggested that red wine is the second major source of human exposure to OTA, following cereals and preceding coffee and beer (Walker, 1999). Red wines had higher concentrations than white ones (Otteneder and Majerus, 2000). Ochratoxin-contaminated wine grape and its products have its major economic impact on the wine industry.

So the aim of this study was to isolate and identify some ochratoxigenic fungi in wine grapes from France. Furthermore, this study was intended to assess the potential for Ochratoxin A contamination of French grapes.

3.3 Materials and methods

3.3.1 French study area

Eight varieties of wine grapes were collected from ten vineyards located in 4 French winemaking regions which are (see figure 3.1): Poitou-Charentes (PC) (vineyard 1), Languedoc-Roussillon (LR) (vineyards 2 to 8), Provence-Alpes Côtes d'Azur (PACA) (vineyard 9), and Alsace (Als) (vineyard 10) and then they were

analyzed. Eight varieties were Ugni Blanc (UB) from PC, Riesling (R) from Als, Cinsault (CN) from PACA and 5 varieties from LR were considered: Sauvignon (SA), Muscat (Mu), Syrah (S), Carignan (CA) and Grenache (G). For the Syrah variety, three areas were considered (a, b and c). All the varieties analyzed except Ugni Blanc, Riesling and Muscat were red vines.

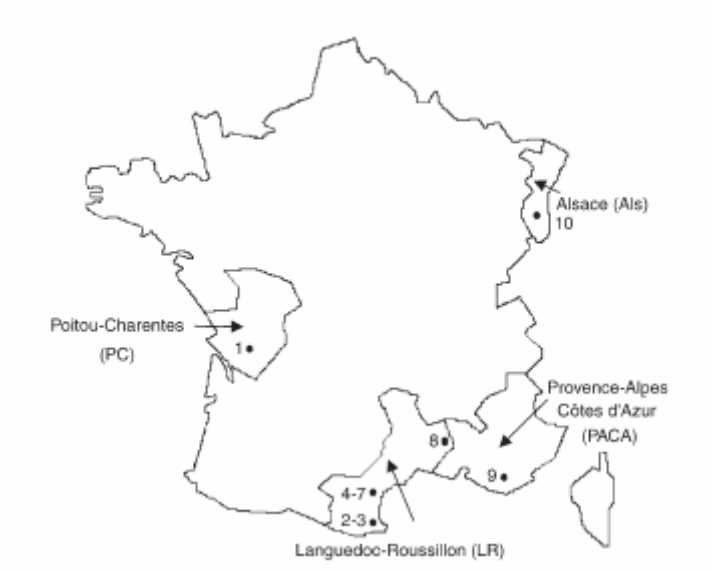

Fig. 3.1 Location of the regions and vineyard studied in France (each number represented one vineyard).

3.3.2 Samples collection

Ten bunches of wine grapes were collected from each vineyard and each growth stages (green berry, early veraison, and ripe berry (harvest time)) by following the two diagonals. Then they were rapidly transported to the laboratory in cool boxes, mycological analyses were immediately done. The remaining samples were frozen at −20 °C for subsequent analysis.

3.3.3 Mycological analysis of grapes

Five berries from each bunch were randomly chosen. Then, they were put onto the surface of the DRBC (Dichloran Rose Bengale Chloramphenicol) agar (Oxoid, Basingstoke, Hampshire, England) (Cahagnier, 1998) and plates were incubated at 25°C for 7 days. Samples were examined daily with a stereomicroscope. *Aspergillus* and *Penicillium* species were isolated and further purified on CZ (CZAPEK Agar, Oxoid) (Cahagnier, 1998) medium and CYA (CZAPEK Yeast extract Agar, Oxoid) medium, respectively. All isolated fungi were identified by morphological characters according to the most accepted criteria of classification (Raper and Fennell, 1965; Klich and Pitt, 1988; Pitt and Hocking, 1997).

3.3.4 Ochratoxigenic ability of the isolates

OTA production ability of 820 isolates was tested on CYA using the method described by Bragulat et al. (2001). Ten milliliter of melted agar was added to 55-mm Petri dishes. Each isolate was inoculated into the center of solidified agar and incubated at 25°C for 7 days. Three parts of cultured agar plugs (inner, middle, and outer area, diameter 5 mm) were removed. Plugs were weighed and introduced into a small vial. A volume of 1.0 ml of methanol was added. After 60 min, the extracts were centrifuged at 10,000 g for 20 min. The supernatant was recuperated, then filtered (Millex®HV PVDF 0.45µm, Millipore) and injected into the HPLC. OTA was analyzed and ochratoxigenic potential was expressed as µg g^{-1} CYA.

3.3.5 Analysis by HPLC (Awad et al., 2005; Bejaoui et al., 2006)

OTA was detected and quantified by reversed-phase HPLC. The HPLC apparatus consisted of a solvent delivery system with fluorescence detector (λ_{ex}=332 nm; λ_{em}=466 nm). The analytical column used was a 150x4.6 mm Uptisphere 5 µm C18 ODB fitted with a guard column of 10x4 mm. The column temperature was 30°C. An aliquot of sample (80 µl) was injected using an auto-injector (BIO-TEK, Milan, Italy). The mobile phase was acetic acid in water 0.2% (A) and acetonitrile (B) delivered at flow rate of 1 ml min^{-1}. Analysis of OTA produced by grape isolates was done using a run time of 20 min and an isocratic method [A (59%)-B (41%)]. OTA was detected by comparing the elution time with the standards (Sigma Aldrich, Steinheim, Germany) and quantified by measuring peak area according to a standard curve. The detection limit was 0.025 µg l^{-1}. All analysis were done in triplicate.

3.3.6 Statistical analysis

The within laboratory relative standard deviation of repeatability (RSD_r, within batch precision) were calculated using a one-way analysis of variance (ANOVA) approach with Statistical Analysis System (SAS Institute, Inc., 1995) and mean values comparison was conducted by Duncan's Multiple Range Test (DMRT).

3.4 Results and discussion

3.4.1 Mycological analysis of grapes

From this study, the predominance fungi of French grapes were observed. Among total, 80% are belonging to the *Aspergillus* genus following by the *Penicillium* genus (20%). *Aspergillus* and *Penicillium* genera were also isolated as the

potential OTA producers in various foodstuffs (Varga et al., 1996). Among *Aspergillus* species, the black aspergilli were the most common (99%). For the remaining aspergilli (1%), isolates of *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus fumigatus* were found. Black aspergilli were represented by three populations: *Aspergillus carbonarius*, *Aspergillus japonicus* and *Aspergillus niger* aggregate. *Aspergillus japonicus* was found as the minority. Moreover, black aspergilli were also found as predominant fungi on Italian (Battilani et al., 2003), Spanish (Belli et al., 2004) and Protuguese (Serra et al., 2003) grapes.

3.4.2 Ochratoxigenic ability of the isolates

The capacity for Ochratoxin A production of all isolates was determined on a solid laboratory medium (CYA) after 7 days at 25°C. According to the ochratoxigenic isolates, results indicated the 93% of the genus *Aspergillus* was compared to 7% of the genus *Penicillium*. Thirty two percent (±1.26) of the total fungal isolates were ochratoxigenic. Among all tested *A. japonicus*, OTA production ability was not more than 20% while the OTA production ability of all isolates of *A. carbonarius* was 71%. Their OTA production ranged from 1.2 to 37.5 µg/g.

For the all cases of *A. niger* aggregate, ochratoxigenic isolates never exceeded 20%.

According to our results, the lower number of *A. japonicus* species, the fewer ochratoxigenic isolates recovered and their low OTA-producing potential showed that *A. japonicus* was unimportant in OTA French grape contamination. The higher number of *A. carbonarius*, the high frequency of ochratoxigenic isolates and their potential to produce OTA showed that this specie is the most OTA producer on grapes

in France. *A. carbonarius* was also reported as the main OTA producer in Spanish (Abarca et al., 2001), Protuguese (Serra et al., 2003) and Italian grapes (Battilani et al., 2003) because of its invasive character to colonize and penetrate berries even without skin damage (Battilani and Pietri, 2002).

3.4.3 Growing season and ochratoxigenic isolates

According to the fungal population during the growing season, at the green berry stage, there are no OTA producer isolates were found. In early veraison, there are a few OTA producer fungi were recovered and predominantly *A. carbonarius*. At harvest time, fungal contamination was highly increased. Approximately, 90% of ochratoxigenic isolates were from ripe berries and 92% were *A. carbonarius*. This has also been observed in Portuguese vineyards (Serra et al., 2003). Additionally, at harvest time, ochratoxigenic *A. niger* aggregate were found and reached 50% of the total ochratoxigenic black aspergilli isolates whereas ochratoxigenic *A. carbonarius* were 48%. At harvest time, grapes will be more ripen than other growing stages. Carbohydrate will be converted to sugar and leads to sugar accumulation and acid discretion. Furthermore, the grapes cuticles will embitter. Thus, fungi from atmosphere can easily penetrate onto the surface of grapes. Fungi will use those sugars for their growth and as the substrate for mycotoxin production. This phenomenon could explain about fungal abundance and development in French grapes.

3.4.4 Regional variation and varieties

According to the regional variation and varieties, the most fungal isolates were from the Languedoc-Roussillon region, followed by CN from Prevence-Alpes Côte d'Azur, R from Alsace and finally UB from Poitou-Charentes. Furthermore, the western (Poitou-Charentes) or eastern (Alsace) vineyards, limited fungal population was found. There are no ochratoxigenic fungi were isolated on the UB variety and only three on Riesling. On those two grapes varieties, OTA never detected. On CN variety, there are 13 ochratoxigenic isolates (*A. niger* aggregates) were found and OTA content in this variety was 0.11 ppb. Accordingly to grapes from Languedoc-Roussillon region, 52% of *A. carbonarius* and 48% of *A. niger* aggregate were found as the majority of ochratoxigenic isolates. The highest OTA concentrations in grapes from this region were observed in SA variety (0.46 ng g^{-1}). Black Aspergilli were found as the large fungal flora in French Mediterranean southern vineyards because of these fungi will be resistant to high sun exposure and to very hot (40°C during summer time) and dry environments with low rainfall levels which is the character of this climate (Serra et al., 2003).

3.5 Conclusion

From this study, we concluded that *A. carbonarius* and *A. niger* aggregates are the most ochratoxin A producer in wine grapes from France. Moreover, *A. japonicus* can also produce a small quantity of ochratoxin A in wine grape. Harvest time is the critical time for ochratoxigenic fungi and OTA contamination. Southern Mediterranean regions especially Languedoc-Roussillon were the most contaminated with OTA.

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CHAPTER IV

ISOLATION OF *Bacillus spp*. FROM THAI FERMENTED SOYBEAN (THUA-NAO): SCREENING FOR AFLATOXIN B_1 AND OCHRATOXIN A DETOXIFICATION

4.1 Abstract

One Aspergillus flavus aflatoxin producing strain and twenty-three isolates of *Bacillus* spp. were isolated from soybean and fresh Thua-Nao collected from the North of Thailand. Inhibition studies of *A. flavus* and *A. westerdijkiae* NRRL 3174 (reference strain) growth by all isolates of *Bacillus* spp. were conducted by dual culture technique on agar plates. These isolates were also tested for AFB_1 and OTA detoxification ability on both solid and liquid media. One *Bacillus* strain was able to inhibit growth of both *Aspergillus* strains and to remove both mycotoxins (decrease of 74% of AFB_1 and of 92.5% of OTA). It was identified by ITS sequencing as *Bacillus licheniformis.* The OTA decrease was due to degradation in OTα. Another *Bacillus* strain inhibiting both *Aspergillus* growth and detoxifying 85% of AFB_1 was identified

as *Bacillus subtilis*. AFB_1 decrease has not been correlated to appearance of a degradation product. The possibility of reduce AFB_1 level by a strain from the natural flora is of great interest for the control of the quality of fermented soybean. Moreover the same strain could be a source of efficient enzyme for OTA degradation in other food or feeds.

4.2 Introduction

Thua-Nao is one of the oldest traditionally fermented soybean products produced for years by the people in many small villages up north of Thailand. *Bacillus* spp., a Gram-positive, strict or facultative aerobe and endospore-forming bacteria, was found to be the dominant microflora of this product (Chantawannakul et al., 2002). However, during fermentation, the soybeans would be sometimes contaminated with toxigenic fungi for which the intrinsic and extrinsic factors may also induce the mycotoxin formation, leading to health risk for consumers (Garcia et al., 1997).

The mycotoxins of the greatest significance in foods and feeds are aflatoxins which are of great concern because of their detrimental effects on the health of humans and animals, including carcinogenic, mutagenic, teratogenic, and immunosuppressive effects (Eaton and Gallangher, 1994). They are produced mainly by *Aspergillus flavus, Aspergillus parasiticus* or *Aspergillus nominus*. The four main naturally produced aflatoxins are B_1, B_2, G_1 and G_2, with B usually being the aflatoxin found at the highest concentration in contaminated food and feed.

Other important mycotoxins which have received increasing interest from both scientific communities and food committees are ochratoxin A (OTA). OTA is

produced by *Aspergillus ochraceus* and related species. *Aspergillus* section *Nigri*, for example *Aspergillus niger* aggregate and *Aspergillus carbonarius*, was shown to be responsible for OTA production in grapes (Abarca et al., 2001; Cabanes et al., 2002). *Penicillium* species, particularly *Penicillium verrucosum* is widely producer as well (Pitt, 1987). OTA is a derivative of isocoumarin linked to L-phenylalanine and it has a potent nephrotoxic (Krogh et al., 1974; Mortensen et al., 1983), teratogenic (Arora and Froelen, 1981), genotoxic (Dirheimer, 1998), immunosuppressive (Haubeck et al., 1981) and carcinogenic (Boorman, 1989) properties. If the consumers take for a long term, moderate to low of these mycotoxin concentrations, much more serious problem may arise.

Some researches showed that *Bacillus subtilis* could be able to inhibit *Aspergillus* growth (Foldes et al., 2000) and that *Bacillus stearothermophilus* could inhibit aflatoxin production by *A. flavus* and *A. parasiticus* (Faraj et al., 1993). Mixing *Bacillus subtilis* with groundnuts could reduce the damage caused by *A. flavus* (Sommartya, 1997). In addition, fermentation of contaminated grains has been shown to degrade aflatoxins (Dam et al., 1977) and Smith and Haran (1993) reported that *Bacillus strearothermophilus* could degrade Aflatoxin B_1 in-vitro. Concerning ochratoxin A, a few microorganisms are able to degrade it, for example *Saccharomyces cerevisiae*, *Lactobacillus* spp., *Bacillus subtilis* and *Bacillus licheniformis* (Bohm et al., 2000), *Rhizopus* (Varga et al., 2005) and *Aspergillus niger* (Bejaoui et al., 2006).

Finally, this study has been then performed to study the interaction between *Bacillus* spp., isolated strains from Thai fermented soybean product, and contaminating *A. flavus* in soybean in order to limit aflatoxin production. The

interaction between *Bacillus* strain and *A. westerdijkiae* NRRL 3174 (ex *ochraceus*) was also studied since this strain is considered as a reference strain for high OTA production. The second objective was to study the detoxification of aflatoxin B_1 (AFB_1) and ochratoxin A (OTA) by *Bacillus* spp. in order to find an efficient strain to remove these toxins.

4.3 Materials and methods

4.3.1 Isolation of *Bacillus* spp. from Thai-fermented soybean product (Thua-Nao)

Three samples of fresh Thua-nao were collected from the local market in Chiangmai and Maehongson province which are located in the North of Thailand. One gram of each sample was suspended in 9 ml of phosphate buffer, mixed well by vortex and agitated for 30 minutes. Appropriate dilutions (dilution of 10^{-7}) were made with phosphate buffer as diluent. One hundred micro-liter of each dilution were spreaded on nutrient agar (3 g beef extract (Difco laboratories, USA), 5 g peptone (Fluka BioChemika), 15 g agar (Difco laboratories, USA) in 1 L of distilled water) and incubated at 37°C for 24 h. Colonies appeared on plate were picked up and maintained on nutrient agar at 4°C. Stock culture were done by cultivation on nutrient broth and stored at –20°C after addition of sterile glycerol at 20%.

4.3.2 Identification of 23 isolates of *Bacillus* spp. by API 50CH system

Twenty three colonies were first characterized by Gram's stain technique and then identified as *Bacillus* spp. by its sugar utilization pattern in the API 50CH system

(API 50 CHB medium, BioMèrieux® SA 673 620 399 RCS Lyon, 69280 Marcy-l'Etoile, France). All isolates were maintained on nutrient agar.

4.3.3 Identification of the interested isolates with ITS sequencing

4.3.3.1 DNA extraction

For identification of two interesting *Bacillus* isolates with ITS sequencing, the method described by Liu et al. (2000) was used. In a 1.5-ml Eppendorf tube containing 500 ml of lysis buffer (400 mmol l^{-1} Tris-HCl [pH 8.0], 60 mmol l^{-1} EDTA [pH 8.0], 150 mmol l^{-1} NaCl, 1% sodium dodecyl sulfate), a small lump of culture broth was added by using a steriled toothpick, with which the lump of culture broth was disrupted. The tube was then left at room temperature for 10 min. After adding 150 µl of potassium acetate (pH 4.8 which is made of 60 ml of 5 mol l^{-1} potassium acetate, 11.5 ml of glacial acetic acid, and 28.5 ml of distilled water), the solution was stirred and centrifuged at the speed of 10,000 *g* for 1 min. The supernatant was transferred to another 1.5-ml Eppendorf tube and centrifuged again as described above. An equal volume of isopropyl alcohol was added into the tube. It was mixed by inversion briefly. The solution was centrifuged at the speed of 10,000 *g* for 2 min and the supernatant was discarded. The DNA pellet was washed in 300 µl of 70% ethanol. After the pellet was spun at 10,000 g for 1 min, the supernatant was discarded. The DNA pellet was air dried and dissolved in 50 µl of 1xTris-EDTA, and 1 µl of the purified DNA was used in 50 µl of PCR mixture.

4.3.3.2 PCR Reaction

PCR was performed on Stratagent Robocycler Gradient 96, 230 VAC, 2A, 50 Hz (Serial Number: 9802258) with the Taq recombinant polymerase (Invitrogen, USA). Amplification was carried out in 50 µl reaction mixture containing: 5 µl of Taq polymerase buffer10X, 1.5 µl of 50 mmol l^{-1} $MgCl_2$, 1 µl of dNTP 10 mmol l^{-1} of each (Promega), 1 µmol l^{-1} of each primer, 1.5 U of Taq, about 50 ng of DNA genomic, H_2O up to 50 µl. Reaction conditions were 94°C for 40 s, 58°C for 40 s and 72°C for 40 s x 35 cycles followed by an incubation at 72°C for 10 min. The amplified products were examined by agarose gel electrophoresis. The *ß-tubulin* gene was used as positive control and primer sequences were 27F: AGAGTTTGATCCTGGCTCAG; 1525R: AAGGAGGTGATCCAGCC, which amplified a 750 bp fragment on genome.

4.3.3.3 Data analysis

The website http://www.ncbi.nlm.nih.gov/entrez/query.fcgi was used for sequence analysis at the GenBank database.

4.3.4 Isolation of *A. flavus* from soybean in Thailand

Eight packs of soybean seed were purchased from local market in Chiangmai and Maehongson province. One hundred soybean seeds of each pack were randomly sampled. They were then surface rinsed with 10% v/v Clorox for 10-15 minutes, rinsed with sterilized distilled water 3 times, then placed onto sterilized pre-wetted tissue paper into a plastic box and left at the room temperature for 2-3 days. Green colonies of *A. flavus* were picked and maintained on Potato Dextrose Agar (PDA,

Difco laboratories, USA). *A. flavus* was identified by morphological observations according to Pit and Hocking (1997), Varga et al. (2000a), Abarca et al. (2004).

4.3.5 Screening of AFB_1-producing *A. flavus*

AFB_1-producing *A. flavus* isolates were screened by modified method of Bragulat et al. (2001). The culture media used were Yeast Extract - Sucrose (YES) agar, which contained per litre: yeast extract 20 g; sucrose 160 g; agar 16 g. Czapek Dox (CZA) agar, which contained per litre: K_2HPO_4 1 g; Czapek concentrate 50 ml; trace metal solution 1 ml; sucrose 30 g; agar 20 g. Ten milliliter of each melted agar (YES and CZA) were added to 55-mm Petri dishes. Each isolate of *A. flavus* was inoculated into the center of solidified agar and incubated at 25°C for 7 days. Three parts of cultured agar plugs (inner, middle, and outer area, diameter 5 mm) were removed. Plugs were weighed and introduced into a small vial. A volume of 1.0 ml of methanol was added. After 60 min, the extracts were centrifuged at 10,000 g for 20 min. The supernatant was recuperated, then filtered (Millex®HV PVDF 0.45µm, Millipore) and injected into the HPLC.

4.3.6 Screening of the isolated *Bacillus* spp. for growth inhibition of *A. flavus*

Growth inhibition studies of isolated *A. flavus* by all isolates of *Bacillus* spp. were conducted by dual culture technique. The culture media used were nutrient agar and Potato Dextrose Agar (PDA). They were poured into 100-mm Petri-dishes. Then AFB_1-producing *A. flavus* which tested by the method described above was selected and inoculated in the center of solidified agar and then each isolate of *Bacillus* spp.

was inoculated at 10-mm far from the side of the Petri-dish. They were then incubated at 25 and 37°C for 7 days. The experiment was carried out in triplicate. Radial growth reduction was calculated as follows: (a-b) / a (where a = radial growth measurement of the pathogen in control and b is that of the pathogen in the presence of bacteria tested); these values were expressed as percentage inhibition of radial mycelial growth.

4.3.7 Screening of the isolated *Bacillus* spp. for growth inhibition of *Aspergillus westerdijkiae* NRRL 3174

A. westerdijkiae NRRL 3174 was chosen as a reference strain for OTA producer *Aspergillus.* Inhibition studies of *A. westerdijkiae* NRRL 3174 by all isolates of *Bacillus* spp. were conducted by dual culture technique. The culture media used were nutrient agar and Czapek Yeast Extract (CYA) agar. The culture media were poured into 100-mm Petri-dishes. Then *A. westerdijkiae* NRRL 3174 was inoculated in the center of solidified agar and then each isolate of *Bacillus* spp. was inoculated at 10-mm far from the side of the Petri-dish. They were incubated at 25 and 37°C for 7 days. The experiment was done in triplicate. Radial growth reduction was calculated as follows: (a-b) / a (where a = radial growth measurement of the pathogen in control and b is that of the pathogen in the presence of bacteria tested); these values were expressed as percentage inhibition of radial mycelial growth.

4.3.8 Degradation of AFB_1 and OTA by 23 isolates of *Bacillus* spp.

Standard AFB_1 and OTA were used through this experiment. One hundred micro liter of *Bacillus* spp. from frozen stock suspension was inoculated in 5 ml of

Nutrient Broth filled in 15 ml Falcon tube. Standard AFB_1 or OTA at initial concentration of 5 mg l^{-1} were added. The cultures of *Bacillus* spp. with toxin were then incubated at 37°C in shaking incubator with speed of 150 rpm for 1 week.

4.3.9 AFB_1 and OTA degradation during growth of the 2 selected isolates (*Bacillus* CM 21 and *Bacillus* MHS 13)

One ml of each frozen *Bacillus* stock culture (*Bacillus* CM 21 and *Bacillus* MHS 13) was transferred to a 250-ml Erlenmeyer flask containing 100 ml of sterilized nutrient broth (NB) and shaking incubated at 37°C for 48 hours. The optical density (OD) of the culture broth was measured by using a spectrophotometer at 660 nm every 6 hours. Then, cultured broth was used to inoculate 100 ml of fresh sterilized NB. The culture was then diluted to an absorbance approximately 0.1 with fresh media. The initial concentration of 5 mg l^{-1} of each standard AFB_1 or OTA were added into NB. All cultures were incubated at 37°C with agitation. Five milliliters of each culture were sampled twice a day for 5 day and then once a day for the following 10 days. Optical density at 660 nm was measured each time of sampling.

4.3.10 Extraction of residual AFB_1 and OTA

4.3.10.1 Aflatoxin B_1

AFB_1 extraction was made according to Sánchez et al. modified method (2005). Ten milliliters of acetone and 15 ml of dichloromethane were added to nutrient broth. Following the addition of each solvent, the cultures were shake-agitated at 150 rpm for 30 min. The filtrate was partitioned in a preparatory funnel

into an aqueous phase and a dichloromethane phase that contained the most of the aflatoxin. The aqueous phase was partitioned again with 15 ml of fresh dichloromethane. The two dichloromethane fractions were combined. Residual H_2O was removed from the final dichloromethane solution with 1g Na_2SO_4 and the solution was filtered with Whatman paper No.1. The filtrate was evaporated at 40°C (90 rpm) until dry. The residue was dissolved with 1 ml acetonitrile, filtered (Millex®HV PVDF 0.45µm, Millipore) and analyzed by HPLC.

4.3.10.2 Ochratoxin A

Thirty four microliters of 12N HCl (NormaPur) was added to the culture followed by 3.5 ml of chloroform (Prolabo, R.P. NormaPur). The solution was partitioned in a preparatory funnel into an aqueous phase and a chloroform phase that contained most of the OTA. The chloroform phase was recuperated and then evaporated under nitrogen gas at 65°C (90 rpm) until dry. The samples were then dissolved in 1 ml methanol (Fisher Scientific), filtered (Millex®HV PVDF 0.45µm, Millipore) and analyzed by HPLC for the OTA residues.

4.3.11 Analysis by HPLC

4.3.11.1 Aflatoxin B_1

The HPLC method for AFB_1 analysis was modified from Chan et al. (2004). The HPLC apparatus consisted of a solvent delivery system with both fluorescence (λ_{ex}=364 nm; λ_{em}=440 nm) and UV detectors (λ=225 nm and 362 nm). The spectra range is from 200 to 500 nm. The analytical column used was a 150 x 4.6 mm

Uptisphere 5 µm C18 ODB fitted with a guard column of 10 x 4 mm. The column temperature was 25°C. An aliquot of sample (80 µl) was injected using an auto-injector (BIO-TEK, Milan, Italy). The mobile phase was 0.1% phosphoric acid (A) and methanol/acetonitrile (50:50) (B) delivered at flow rate of 1 ml min^{-1} for 30 min. The sample was eluted with a linear gradient for 30 min. AFB_1 and its derivatives were detected by comparing the elution time and maximum absorption of UV with the standards.

4.3.11.2 Ochratoxin A (Awad et al., 2005)

The HPLC apparatus consisted of a solvent delivery system with fluorescence detector (λ_{ex}=332 nm; λ_{em}=466 nm). The analytical column used was a 150 x 4.6 mm Uptisphere 5 µm C18 ODB fitted with a guard column of 10 x 4 mm. The column temperature was 30°C. An aliquot of sample (80 µl) was injected using an auto-injector (BIO-TEK, Milan, Italy). The mobile phase was acetic acid in water 0.2% (A) and acetonitrile (B) delivered at flow rate of 1 ml min^{-1} for 45 min. The sample was eluted with a linear gradient from 10% to 50% of B over the first 30 min followed by a linear gradient to 90% of B from 30 to 35 min and then a steady flow of 90% of B through 8 min and then reduced to 10% of B through 2 min. OTA and its derivative products were detected by comparing the elution time with the standards.

4.3.12 Statistical analysis

The within laboratory relative standard deviation of repeatability (RSD_r, within batch precision) for both of the growth inhibition of *A. flavus* and *A.*

westerdijkiae NRRL 3174 and also of the degradation of AFB_1 and OTA by HPLC analysis were calculated using a one-way analysis of variance (ANOVA) approach.

4.4 Results and discussion

4.4.1 *Bacillus* isolation and identification

Three samples of fresh Thua-Nao, respectively two and one of them, were collected from the local market in ChiangMai and Maehongson province which located in the North of Thailand and they were labelled with the code FTN 01, FTN 02 and FTN 03. Twenty three colonies were isolated from these 3 samples on PDA-nutrient agar. Seven and eight isolates of *Bacillus* spp. were isolated from the two samples collected from ChiangMai province and named respectively CM 11 to CM 17 and CM 21 to CM 28. The seven isolates collected on the sample from Maehongson province were named as MHS 11 to MHS 18. Morphological and physiological characteristics of all isolates were identified as *Bacillus* spp. and confirmed by the API 50CH system.

4.4.2 *A. flavus* isolation and screening

Eight packs of soybean seed were purchased from local market in ChiangMai and Maehongson province. One hundred soybean seeds of each pack were randomly sampled for *A. flavus* isolation. Three green colonies of *A. flavus* were picked and maintained on Potato Dextrose Agar (PDA). They were assigned as SD 1 to SD 3 which means seed number 1 to seed number 3. Figure 4.1 shows isolated colony of *A. flavus* on soybean and figure 4.2 shows the colony of *A. flavus* on PDA.

Fig. 4.1 *A. flavus* on Soybean.

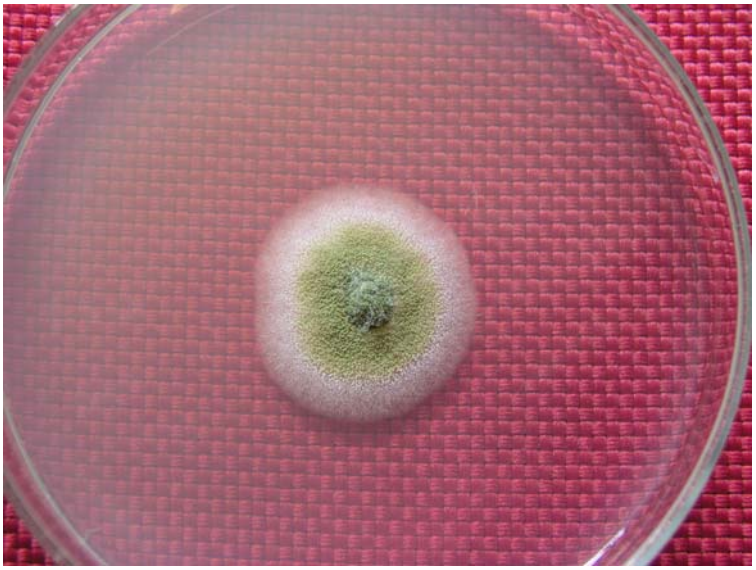

Fig. 4.2 Colony of *A. flavus* on Potato Dextrose Agar (PDA).

All three *A. flavus* isolates (SD 1 to SD 3) were tested for aflatoxin B_1 production ability on YES and CZA medium according to the method described by Bragulat et al. (2001). Results of AFB_1 production on both media analysis by HPLC showed that only *A. flavus* SD 1 is AFB_1 producer.

4.4.3 Screening of the isolated *Bacillus* and identification

4.4.3.1 Screening of the isolated *Bacillus* spp. for growth inhibition of *A. flavus* and *A. westerdijkiae* NRRL 3174

Twelve isolates out of twenty three isolates of *Bacillus* spp. could inhibit the growth of *A. flavus* (data not shown). The average percentage of inhibition was 52%. According to these results, the growth of *A. flavus* was inhibited by *Bacillus* MHS 13 more than other isolates. Regarding to the results for *A. westerdijkiae* NRRL 3174, eight isolates can inhibited its growth which the average percentage of inhibition is 34%. Moreover, *Bacillus* CM 21 is responsible for growth inhibition of *A. westerdijkiae* NRRL 3174 more than other isolates.

4.4.3.2 Screening of the isolated *Bacillus* spp. for removal of AFB_1 and OTA

The 23 isolates were tested for AFB_1 and OTA removal. Results are shown in figure 4.3 and 4.4 respectively. Most of the strains were able to detoxify Aflatoxin but only some of them could detoxify OTA. After one week at least 60% AFB_1 was degraded by eleven *Bacillus* strains. Among them CM 17, CM 23 and MHS 13 were the most efficient. The highest percentage of OTA removal was obtained by *Bacillus* CM 21: 82%. Since this last strain was able to degrade more than 60% AFB_1 it has been retained like strain MHS 13 for the continuation of work.

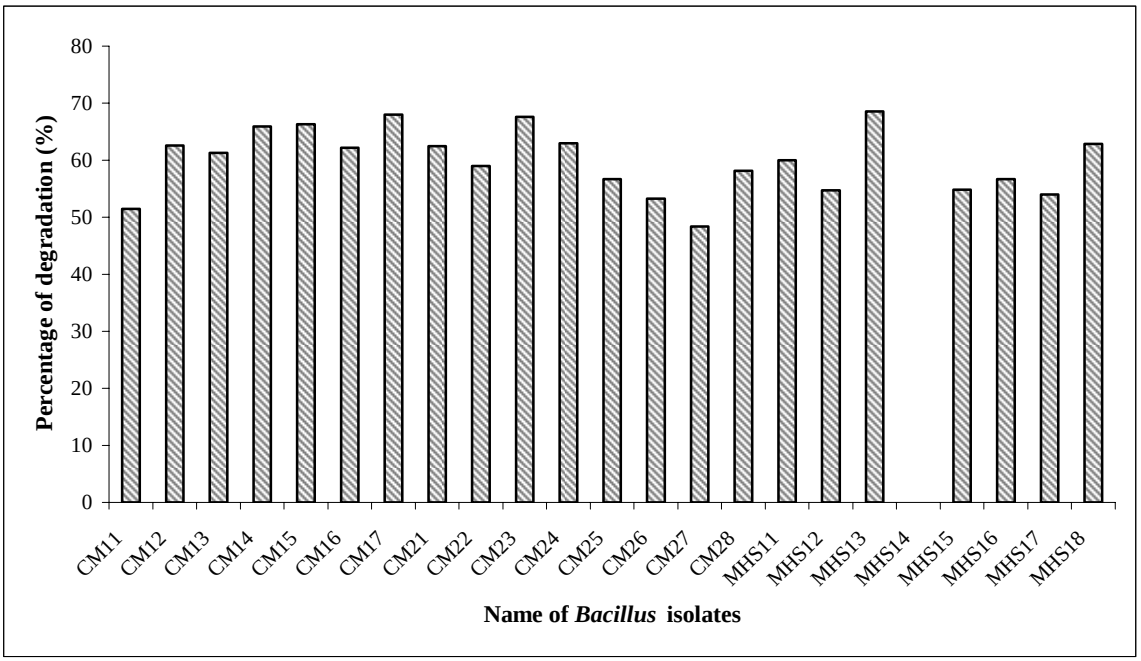

Fig. 4.3 Percentage of AFB_1 degradation by all isolates of *Bacillus* spp.

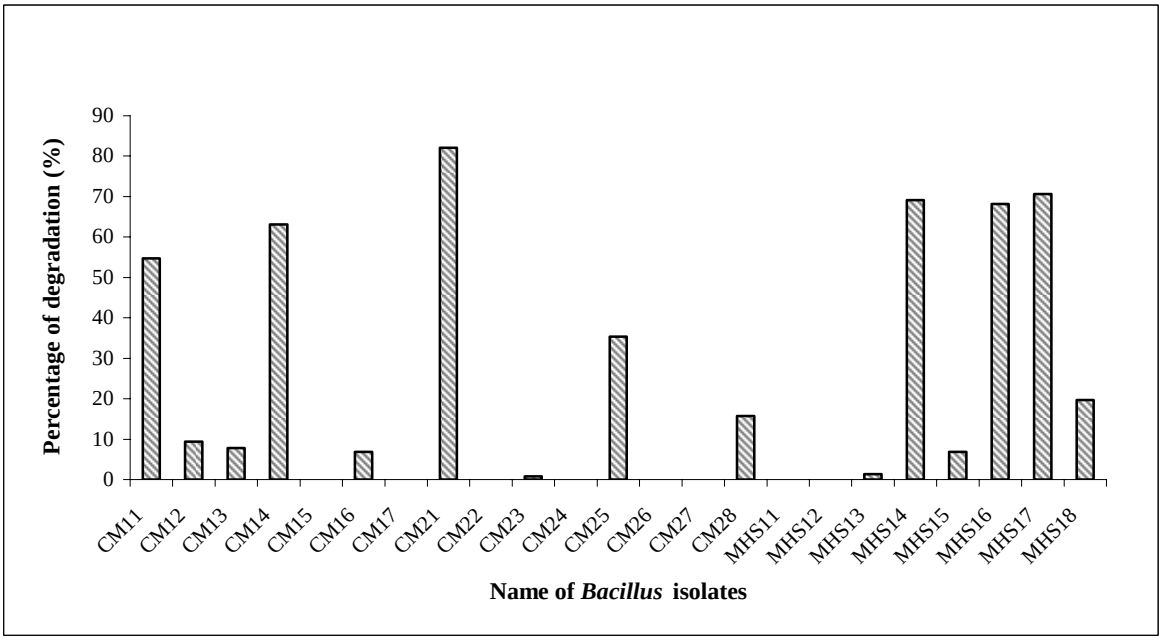

Fig. 4.4 Percentage of OTA degradation by all isolates of *Bacillus* spp.

4.4.3.3 Identification of two interesting isolates with ITS sequencing

The two interesting *Bacillus* isolates, *Bacillus* CM 21 and *Bacillus* MHS 13, were identified by ITS sequencing. The results of sequence analysis with GenBank database showed that *Bacillus* CM 21 is *Bacillus licheniformis* (99% identity) and *Bacillus* MHS 13 is *Bacillus subtilis* (100% identity).

4.4.4 Kinetics AFB_1 and OTA removal during growth of the 2 selected isolates (*B. licheniformis* CM 21 and *B. subtilis* MHS 13)

Detoxification kinetics of AFB_1 and OTA in *Bacillus* CM 21 and *Bacillus* MHS 13 liquid culture was investigated. We could observe that AFB_1 decreased during the first 5 to 6 days (Figure 4.5 and 4.6). In addition, the percentage of AFB_1 removal by *Bacillus* CM 21 and *Bacillus* MHS 13 was respectively 74% and 85%.

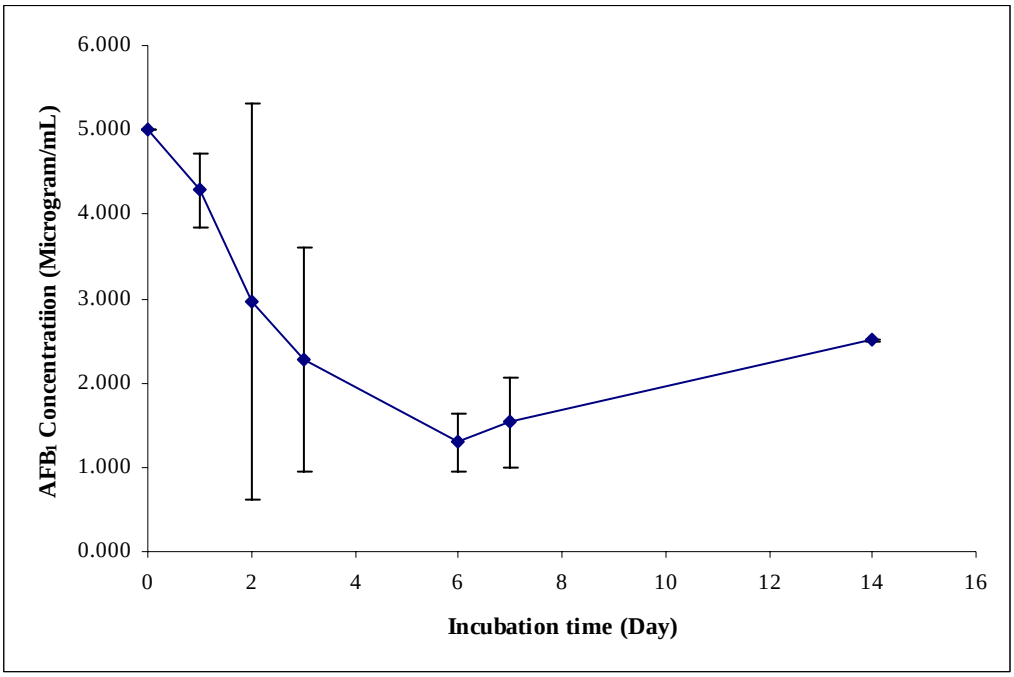

Fig. 4.5 AFB_1 degradation during the growth of *Bacillus* CM 21.

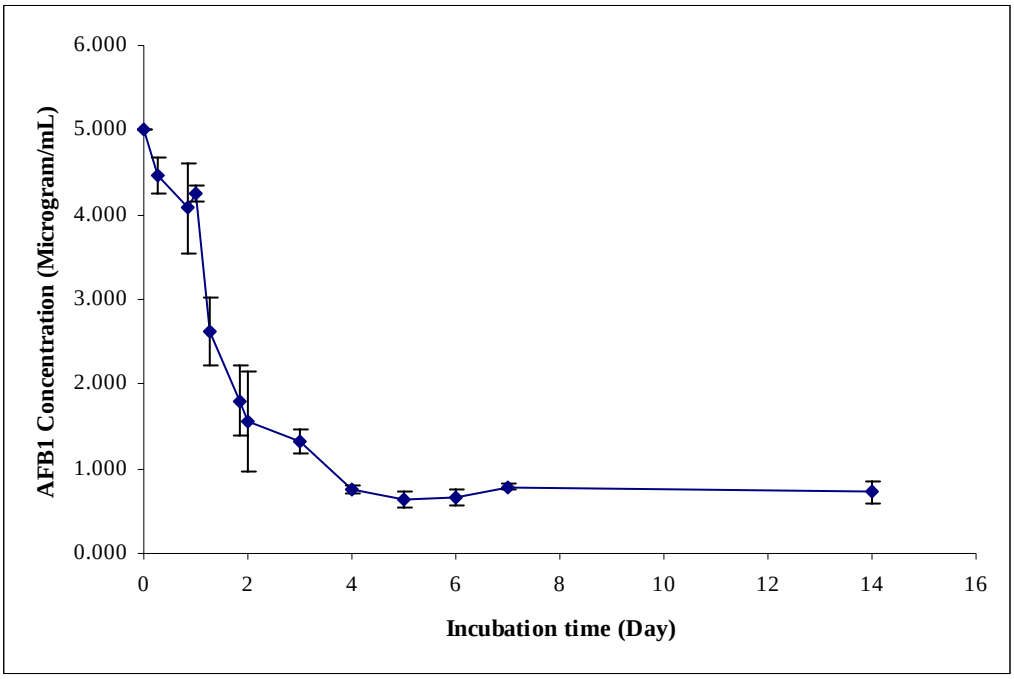

Fig. 4.6 AFB_1 degradation during the growth of *Bacillus* MHS 13.

Regarding to OTA degradation (figure 4.7), *Bacillus* CM 21 can degrade OTA within 48 hours of incubation while ochratoxin α was increasing during the same time (data not shown). The percentage of OTA degradation by this isolate was 92.5%.

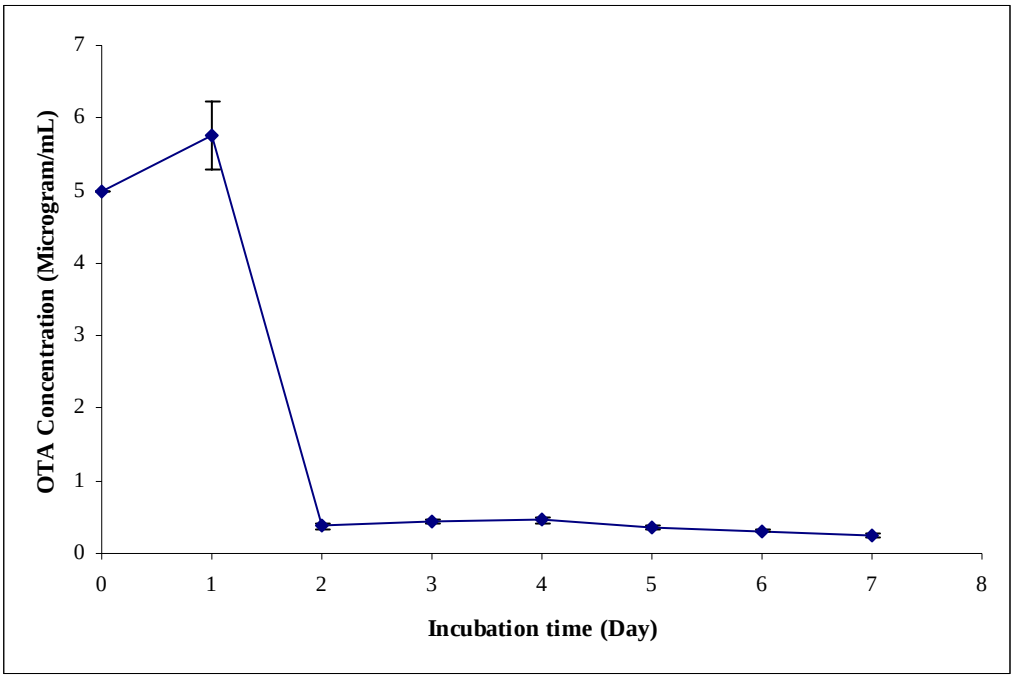

Fig. 4.7 OTA degradation during the growth of *Bacillus* CM 21.

On the opposite, there was no OTA degradation during the growth of *Bacillus* MHS 13 as observed in previous experiment on agar plates. Table 4.1 shows some characteristics of *Bacillus* growth for both strains: duration of growth phase and produced ammonium.

Table 4.1 Some characteristics for *Bacillus* growth in liquid medium.

Strain	Duration of growth phase (h)	Time for maximal production of ammonium (h)	Maximal concentration of ammonium (mg l^{-1})
B. licheniformis CM 21	36	30	220
B. subtilis MHS 13	6	20	200

In this study, *Bacillus* spp. was investigated for *Aspergillus* growth inhibition for mycotoxins producing strains and for aflatoxin B_1 and OTA reduction ability. *Bacillus* spp., a Gram-positive, strict or facultative aerobe and endospore-forming

bacteria, was found to be the dominant microflora of Thai fermented soybean (Thua-Nao) (Chantawannakul et al., 2002). These fermentations are characterized by extensive hydrolysis of protein to amino acids, peptides and ammonia and a rise of the pH. The pH increase observed during fermentation presumably resulted from proteolysis and the release of ammonia due to utilization of amino acids for growth (Terlabie et al., 2006).

The chemical approach to the detoxification of aflatoxins that have received considerable attention is ammoniation (Smith and Haran, 1993; Bhatnagar et al., 2002). Ammoniation under appropriate conditions results in a significant reduction in the level of aflatoxins in contaminated peanut and cottonseed meals. The mechanism for this action appears to involve hydrolysis of the lactone ring and chemical conversion of the parent compound aflatoxin B_1 to numerous products that exhibit greatly decreased toxicity. Two major products identified as compounds with molecular weight 286 (aflatoxin D_1) and molecular weight 206, have been isolated and tested in various biological systems (Park et al., 1988). The first step in the reaction is reversible if the ammoniation process is carried out under mild conditions. This is a disadvantage of the method. However, when the reaction is allowed to proceed past the first step, the products formed do not revert back to aflatoxin B_1. Reaction products of ammoniation are dependent on temperature, pressure, and the source of ammonia. This method is quite energy consuming and presents some risks from security point of view.

In order to find biological alternative methods microorganisms (including yeasts, mold, and bacteria) have been screened for their ability to modify or inactivate aflatoxins. *Flavobacterium aurantiacum* (NRRL B-184) was shown to remove

aflatoxin from a liquid medium significantly without the production of toxic by-products (Ciegler et al., 1966). The same investigators also found that certain acid-producing molds could catalyze the hydration of aflatoxin B_1 to B_{2a} (a less toxic product). In the case of some *Aspergillus* strains that can be at the same time aflatoxin producer and able to degrade it, peroxydases activities where shown to catalyse aflatoxin degradation (Smith and Haran, 1993). For *Bacillus* strains this has never been reported. In our case, no degradation product of AFB_1 could be detected by HPLC analysis. Growth and ammonium production occurred during the first hours of culture while AFB_1 decrease took several days. So the mechanism remains unknown.

Regarding OTA, several reports describe OTA degrading activities of the microbial flora of the mammalian gastrointestinal tract including rumen microbes of cow and sheep (Galtier and Alvinerie, 1976; Hult et al., 1976; Park et al. 1988) and microbes living mainly in the caecum and large intestine of rats (Madhyastaha et al., 1992). The human intestinal microflora can also partially degrade OTA (Akiyama et al., 1997). The species responsible for OTA detoxification have not yet been identified, although mainly protozoa were suggested to take part in the biotransformation process in ruminants (Kiessling et al., 1984). Degradation of OTA was observed in milk due to the action of *Lactobacillus*, *Streptococcus* and *Bifidobacterium* spp. (Skrinjar et al., 1996), while two other bacteria, *Acinetobacter calcoaceticus* (Hwang and Draughon, 1994) and *Phenylobacterium immobile* (Wegst and Lingens, 1983) could also convert OTA to the much less toxic ochratoxin α in liquid cultures. Furthermore, recent reports describe the OTA degrading activities of some *Aspergillus* and *Pleurotus* isolates and/or their enzyme (Varga et al., 2000b). In our case we observed that OTA is also efficiently detoxified by some *Bacillus* isolates

and especially *B. licheniformis* CM 21 and this has not been reported before for this specie. Similarities between OTA degradation kinetics by *Aspergillus niger* and *Bacillus* isolates and the detection of the degradation product, ochratoxin α, in the ferment broth of *B. licheniformis* suggest that a carboxypeptidase A activity may be responsible for OTA decomposition by these isolates.

The possibility of reduce AFB_1 level by a strain of *B. licheniformis* from the natural flora is of great interest for the control of the quality of fermented soybean. Moreover the same strain could be a source of efficient enzyme for OTA biodegradation in other food or feeds. Accordingly, further studies are in progress to determine which enzymes take part in the detoxification process in *Bacillus* isolates, and to establish the absence of by-products and with any residual toxicity, and probably also genetic modification, are required to harness the potential of these strains.

4.5 Conclusion

From this study, we can concluded that *Bacillus licheniformis* (*Bacillus* CM 21) and *Bacillus subtilis* (*Bacillus* MHS 13), which are the starter culture in Thai fermented soybean product (Thua-Nao), have the ability to detoxified aflatoxin B_1 and ochratoxin A in sold medium as well as in liquid medium. In addition, the percentage of degradation indicated that these are the efficiently strains for aflatoxin B_1 and ochratoxin A removal.

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CHAPTER V

MYCOTOXINS (AFLATOXIN B_1 AND OCHRATOXIN A) DEGRADATION BY *Bacillus* spp.: ISOLATED STRAINS FROM THAI FERMENTED SOYBEAN PRODUCT (THUA-NAO)

5.1 Abstract

Culture supernatant and cellular extract from both *Bacillus licheniformis* and *Bacillus subtilis*, isolated strains from Thai fermented soybean product (Thua-Nao), were tested for aflatoxin B_1 (AFB_1) and ochratoxin A (OTA) detoxification ability in liquid medium containing 10 mg l^{-1} of one of these toxins. *Bacillus licheniformis* showed an extracellular OTA-degradation activity. The OTA decrease was due to the degradation in ochratoxin alpha (OTα). The percentage of OTA degradation by a culture supernatant of this strain was 97.5% in nutrient broth (NB). Optimum activity was found at pH 7.0 and 37°C after 24 h culture incubation time for *Bacillus* and 2 h contact time with OTA. Moreover, OTA was also significantly degraded (69%) by culture supernatant from *Bacillus subtilis* at pH 5.0 and 37°C with 48 h culture incubation time and 2 h contact time.

On the contrary, AFB_1 could be degraded by neither culture supernatant nor cellular extract from these microorganisms. The possibility of AFB_1 reduction demonstrated during growth of the strains may be due to a non-enzymatic mechanism.

Thus some *Bacillus* strains could be a potential control of fermented soybean product quality and OTA detoxification tool in other food or feeds.

In some case of co-contamination by both toxins this tool could be particularly interesting.

5.2 Introduction

Mycotoxins are a large and diverse group of secondary metabolites produced by a range of filamentous fungi, in particular, species of *Aspergillus*, *Penicillium* and *Fusarium* (Smith and Moss, 1985). Mycotoxins are non-antigenic compounds of low molecular weight which occur primarily in cereals and oil seeds and products derived from them. They may be among the world's most significant food contaminants (Fischbach and Rodricks, 1973).

In recent years, the attention of most mycotoxicologists has been focused to a great extent on the continuing quest for better ways to control exposure to aflatoxins, resolution of the questions relating to aflatoxin's acute and chronic toxicity for human. Other important mycotoxins which the International Program on Chemical Safety/World Health Organization has recently selected are ochratoxin because of the most threatening effects are their nephrotoxicity and carcinogenicity. It is one of the mycotoxins for which sufficient new information indicates a need for further study and evaluation with respect to human health potential.

Some toxic metabolites, mycotoxins, were found to occur naturally on foods and feeds from cereals (corn, barley, grain sorghum, oats, rice, rye, wheat), pulses, legumes, soybean, peanut, etc. This chapter, we will emphasize particularly on soybean and its products.

Soybean is one of the oldest crops of the Far East. Soybeans are rich in nutrients such as essential fatty acids, calcium, iron, zinc, fat-soluble vitamins, anticarcinogens, and also extremely rich in a unique group of phytoestrogens called isoflavones. Moreover, most of Thai people who live in many small villages up north of Thailand always used soybean as raw material for the production of one of the oldest traditionally non-salted fermented soybean products called Thua-Nao. There is some experimental data which is un-peer review indicated that after fermentation of Thua-Nao both of AFB_1 and OTA could not be detected in finished product even though the raw materials (soybeans) would be contaminated with *Aspergillus* spp. Further, Rustom (1997) found that soybeans from Argentina were contaminated by *Aspergillus flavus* and *Aspergillus parasiticus* which the intrinsic and extrinsic factors may also induced the mycotoxin formation. Moreover, the same investigator also showed that fourteen of thirty five soybean samples from USA were contaminated by aflatoxin B_1 (less than 20 µg/kg) as well as nine of ninety four soybean samples from Argentina.

Nevertheless, Park et al. (2003) also demonstrated that the content of aflatoxins in meju (crushed Korean soybean cake) decreased to 10-20% of the original level of the toxins after 2 month of their ripening process in brine. The degradation rate of AFG_1 was much faster than that of AFB_1.

Thus, there is the possibility that *Bacillus* spp., a Gram-positive, strict or facultative aerobe and endospore-forming bacteria, dominant microflora of this product (Chantawannakul et al., 2002) play a major role in mycotoxin detoxification or degradation in this fermented soybean products.

Finally, this study has been then performed to study the degradation ability and mechanism of aflatoxin B_1 and ochratoxin A by culture supernatant and cellular extract from *Bacillus* spp.; isolated strains from Thai fermented soybean product (Thua-Nao). Moreover, the second objective was to find the optimal condition for aflatoxin B_1 and ochratoxin A degradation.

5.3 Materials and methods

5.3.1 Bacteria and mycotoxins

Two strains of *Bacillus* spp. isolated from Thai Thua-Nao and as *Bacillus licheniformis* (*Bacillus* CM 21) and *Bacillus subtilis* (*Bacillus* MHS 13) were used through this study (cf chapter IV). They were stored at -20°C in nutrient broth added with 20% of glycerol. Standard aflatoxin B_1 (AFB_1) and ochratoxin A (OTA) were obtained from Sigma Aldrich.

5.3.2 Kinetics growth of *Bacillus* CM 21 and *Bacillus* MHS 13

The frozen *Bacillus* CM 21 and *Bacillus* MHS 13 cultures were reactivated by successive cultivation steps in sterilized nutrient broth (NB). Sterilization was performed at 121°C for 15 min. For inoculation, approximately of 20 ml of a 48 h culture broth was transferred to 250 ml of new sterilized NB. Inoculated cultures were grown by incubation at 37°C and agitation at speed of 120 rpm for 48 h using a Gyrotary shaker incubator (Classic series, New Brunswick Scientific Co., New Jersey, USA). Optical density (at 660 nm) and ammonia production by an enzymatic mmethod (Megazym K-AMAI 10/04, Ireland) were measured during the growth of these cultures.

5.3.3 Preparation of culture supernatant and cellular extract from these strains of *Bacillus* cultures. (figure 5.1)

5.3.3.1 Culture supernatant preparation

The culture supernatant was harvested after different incubation time (0, 8, 24, 28, 32 and 48 hours) by culture centrifugation (Sigma Laboratory Centrifuge, 4K15, Bioblock Scientific, Germany) at 4°C using 5,000 rpm for 15 min. Supernatant from the centrifugation step was filtered aseptically using sterilized cellulose pyrogen free disposable filters of 0.2 µm pore size (Schleicher and Schuell, Germany).

5.3.3.2 Cellular extract preparation

After cultures centrifugation, the supernatant was separated. Then, the pellets were washed twice with sterilized distilled water. The cell pellets were resuspended in phosphate buffer (pH 7.0) in preparation for cell rupture. This suspension was disintegrated by using the sonicator (Bioblock Scientific, VC 72405, 100 watt, Paris, France). The conditions of sonicator were amplitude: 80, minuteur: 1 min by 1 min (Total = 6 min), and pulseur: position 2. The temperature during this disruption step should not more than 20°C and should be carried out on ice to ensure low temperature conditions required for most enzymes. The disintegrated cell suspension was centrifuged at 5,000 rpm for 15 min at 4°C (Sorvall RC 26 Plus, Kendro Lab., Bad Homburg, Germany). Supernatant from the centrifugation step was filtered aseptically using sterilized cellulose pyrogen free disposable filters of 0.2 µm pore size (Schleicher and Schuell, Germany).

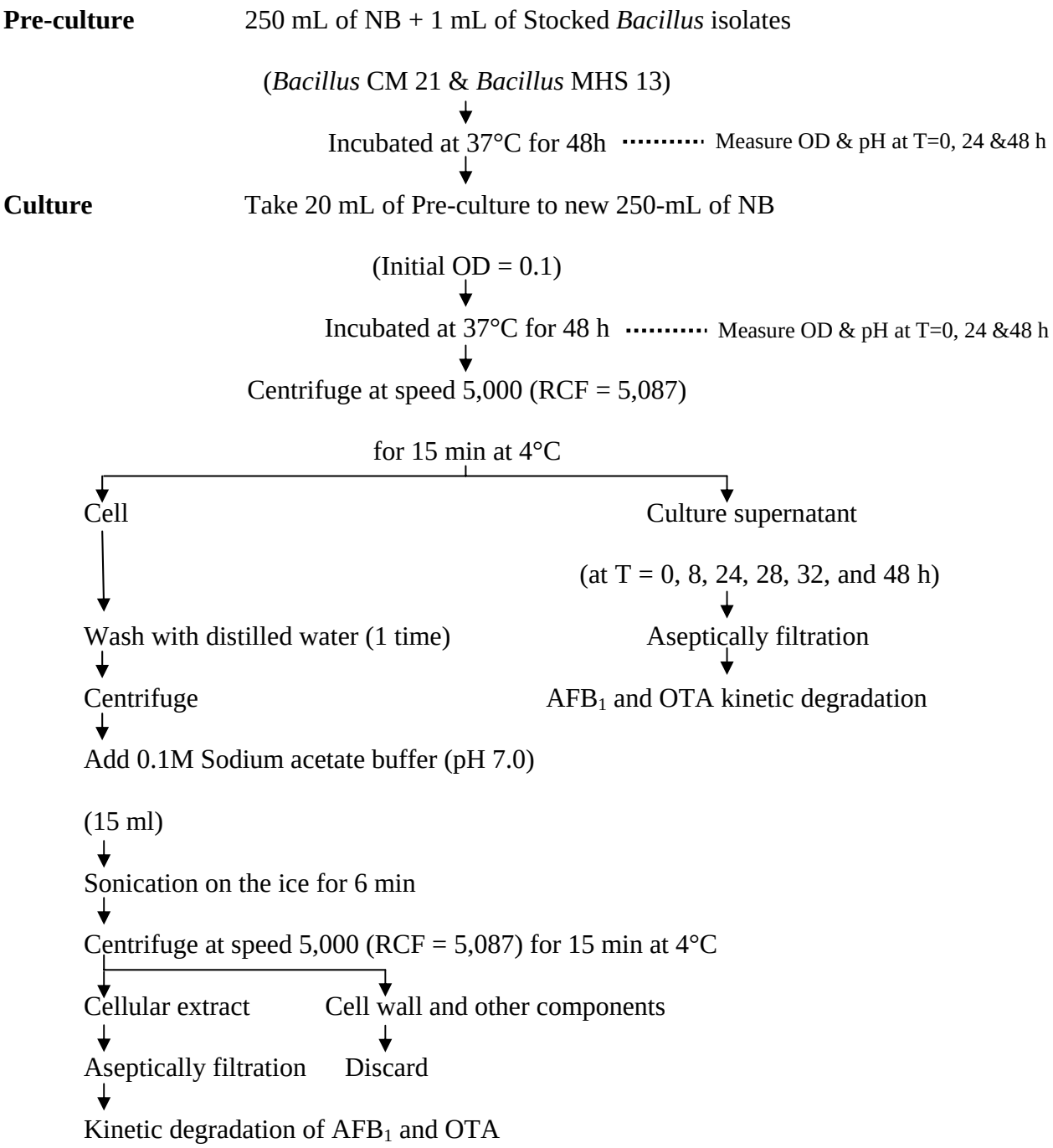

Fig. 5.1 Schematic diagram for culture supernatant and cellular extract preparation from both *Bacillus* CM 21 and *Bacillus* MHS 13.

5.3.4 Kinetic degradation of AFB_1 and OTA by both culture supernatant and cellular extract from these selected strains. (figure 5.2)

5.3.4.1 Degradation of AFB_1 and OTA by culture supernatants

The experiment was performed in 2-ml-Eppendorf tubes in a final volume of 1,000 µl and an initial AFB_1 and OTA-concentration of 10 mg l^{-1}. This involved the addition of 50 µl of each stock solution to 600 µl culture supernatant and 350 µl of 0.1M sodium acetate buffer (pH 5.0 and 7.0). The mixture was incubated in the dark at 37°C without shaking for 0, 15, 30, 45, 60 and 120 min for the optimal reaction and incubation time studies. All experiments were carried out in duplicates. Each experiment was terminated by the addition of 500 µl of HPLC grade chloroform (Prolabo, R.P. NormaPur) for extraction of the remaining AFB_1 and OTA.

5.3.4.2 Degradation of AFB_1 by cellular extracts

The experiment was also performed in 2-ml-Eppendorf tubes in a final volume of 1,000 µl and an initial AFB_1-concentration of 10 mg l^{-1}. This involved the addition 50 µl of each stock solution to 600 µl cellular extract content which containing 350 µl of 0.1M sodium acetate buffer (pH 5.0 and 7.0). The mixture was incubated in the dark at 37°C without shaking for 0, 15, 30, 45, 60 and 120 min for the optimal reaction and incubation time studies. All experiments were carried out in duplicates. Each experiment was terminated by the addition of 500 µl of HPLC grade chloroform (Prolabo, R.P. NormaPur) for extraction of the remaining AFB_1 and OTA.

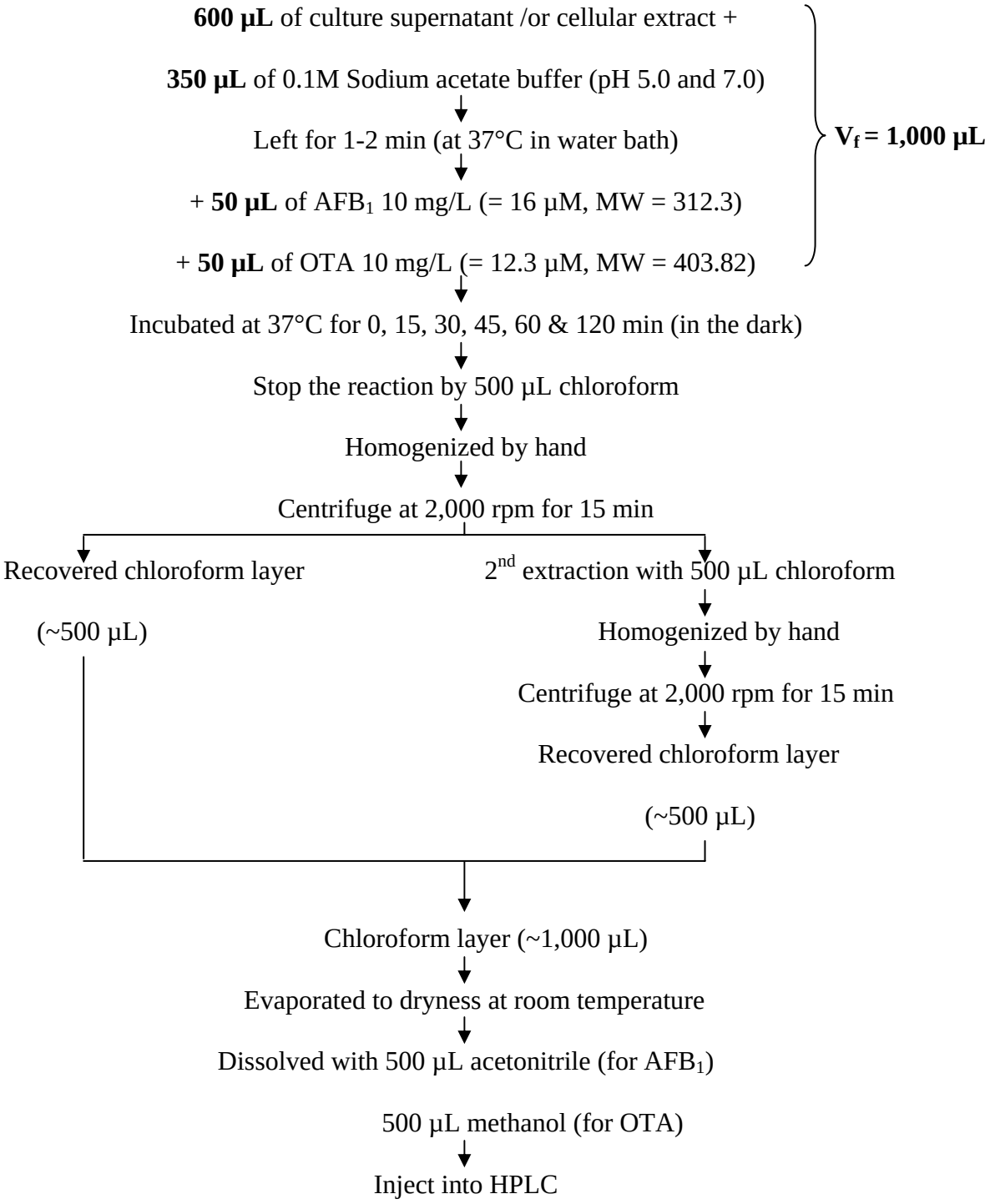

Fig. 5.2 Summarized diagram for kinetic degradation of AFB_1 and OTA by culture supernatant /or cellular extract from both *Bacillus* CM 21 and *Bacillus* MHS 13.

5.3.5 Extraction and quantification of residual AFB_1 and OTA

AFB_1 and OTA extraction was made according to Marisa et al. (2003). They were extracted two times with HPLC grade chloroform (Prolabo, R.P. NormaPur). The lower chloroform layer, obtained by centrifugation at 2,000 rpm for 15 min, was recovered. The chloroform was evaporated until dryness under nitrogen gas, the samples were dissolved in 500 µl acetonitrile (Fisher Scientific), filtered (Millex®HV PVDF 0.45µm 4mm, Millipore) and analyzed by HPLC.

5.3.6 Analysis by HPLC

5.3.6.1 Aflatoxin B_1

The HPLC method for AFB_1 analysis was modified from Chan et al. (2004). The HPLC apparatus consisted of a solvent delivery system with both fluorescence (λ_{ex}=364 nm; λ_{em}=440 nm) and UV detectors (λ=225 nm and 362 nm). The spectra range is from 200 to 500 nm. The analytical column used was a 150x4.6 mm Uptisphere 5 µm C18 ODB fitted with a guard column of 10x4 mm. The column temperature was 25°C. An aliquot of sample (80 µl) was injected using an auto-injector (BIO-TEK, Milan, Italy). The mobile phase was 0.1% phosphoric acid (A) and methanol/acetonitrile (50:50) (B) delivered at flow rate of 1 ml/min for 30 min. The sample was eluted with a linear gradient for 30 min. AFB_1 and its derivatives were detected by comparing the elution time and maximum absorption of UV with the standards.

5.3.6.2 Ochratoxin A (Awad et al., 2005)

The HPLC apparatus consisted of a solvent delivery system with fluorescence detector (λ_{ex}=332 nm; λ_{em}=466 nm). The analytical column used was a 150x4.6 mm Uptisphere 5 µm C18 ODB fitted with a guard column of 10x4 mm. The column temperature was 30°C. An aliquot of sample (80 µl) was injected using an auto-injector (BIO-TEK, Milan, Italy). The mobile phase was acetic acid in water 0.2% (A) and acetonitrile (B) delivered at flow rate of 1 ml/min for 45 min. The sample was eluted with a linear gradient from 10% to 50% of B over the first 30 min followed by a linear gradient to 90% of B from 30 to 35 min and then a steady flow of 90% of B through 8 min and then reduced to 10% of B through 2 min. OTA and its derivative products were detected by comparing the elution time with the standards.

5.3.7 Statistical analysis

The within laboratory relative standard deviation of repeatability (RSD_r, within batch precision) for kinetic degradation of AFB_1 and OTA by both culture supernatant and cellular extract at different conditions from these selected strains were calculated using a one-way analysis of variance (ANOVA) approach with Statistical Analysis System (SAS Institute, Inc., 1995) and mean values comparison was conducted by Duncan's Multiple Range Test (DMRT).

5.4 Results and discussion

In a previous part of this work (cf chapter IV), *Bacillus* spp., a Gram-positive, strict or facultative aerobe and endospore-forming bacteria, was found to be the dominant microflora of Thai fermented soybean (Thua-Nao) accordingly to the

literature of Chantawannakul et al. (2002). These fermentations are characterized by extensive hydrolysis of protein to amino acids, peptides and ammonia and a rise of the pH. The pH increase observed during fermentation presumably resulted from proteolysis and the release of ammonia due to utilization of amino acids for growth (Terlabie et al., 2006). These strains have been selected because of the results in a previous study (see also chapter IV); they exhibited the detoxification ability of AFB_1 and/or OTA during cultures in nutrient broth. Table 5.1 shows some characteristics of *Bacillus* growth for both strains: duration of growth phase and production of ammonium.

Table 5.1 Some characteristics for *Bacillus* growth in liquid medium.

Strain	Duration of growth phase (h)	Time for maximal production of ammonium (h)	Maximal concentration of ammonium (mg l^{-1})
B. licheniformis CM 21	36	30	220
B. subtilis MHS 13	6	20	200

In this work, we study the detoxification ability of both culture supernatant and cellular extract for both strains at different culture time.

5.4.1 Degradation of AFB_1 and OTA by culture supernatants

According to OTA removal, our results showed that culture supernatant from *Bacillus* CM 21 could be efficiently degraded ochratoxin A during log phase. The

degradation product, OTα, could be detected from HPLC (data not shown). The OTA was decreased significantly ($p<0.0001$). The percentage of OTA degradation by culture supernatant of this strain is 97.5% in Nutrient broth (NB). Optimum activity was found at pH 7.0 and 37°C with 24 h culture incubation time and 2 h contact time. Figure 5.3 shows the growth, pH and kinetic degradation of OTA by culture supernatant from *Bacillus* CM 21 in optimal conditions.

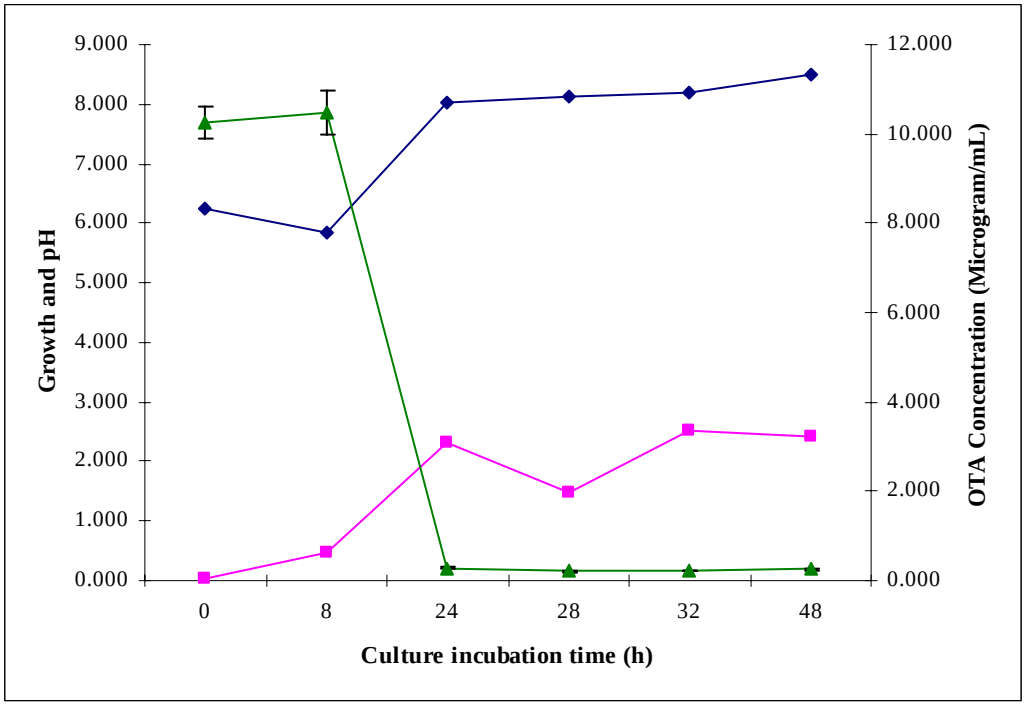

Fig. 5.3 Growth (■), pH (◆) and OTA kinetic degradation (▲) by culture supernatant from *Bacillus* CM 21 at pH 7 after 2 h contact time

The pH value (5.0 and 7.0) has a significant effect on OTA detoxification since we observed that at pH 5.0 the OTA detoxification was half lesser than at pH 7.0 (data not shown). pH 7.0 is also the optimal pH for the growth of *Bacillus* spp. We could note that the maximal degradation activity was reached during the exponential

growth phase of *Bacillus* spp., at 24 hours. We can suppose that more extracellular enzyme has been accumulated at that time.

In the case of culture supernatant from *Bacillus* MHS 13, it could also degrade ochratoxin A significantly ($p<0.0017$) into ochratoxin α. The percentage of OTA degradation by its culture supernatant is 69% as shown in figure 5.4. for optimal conditions.

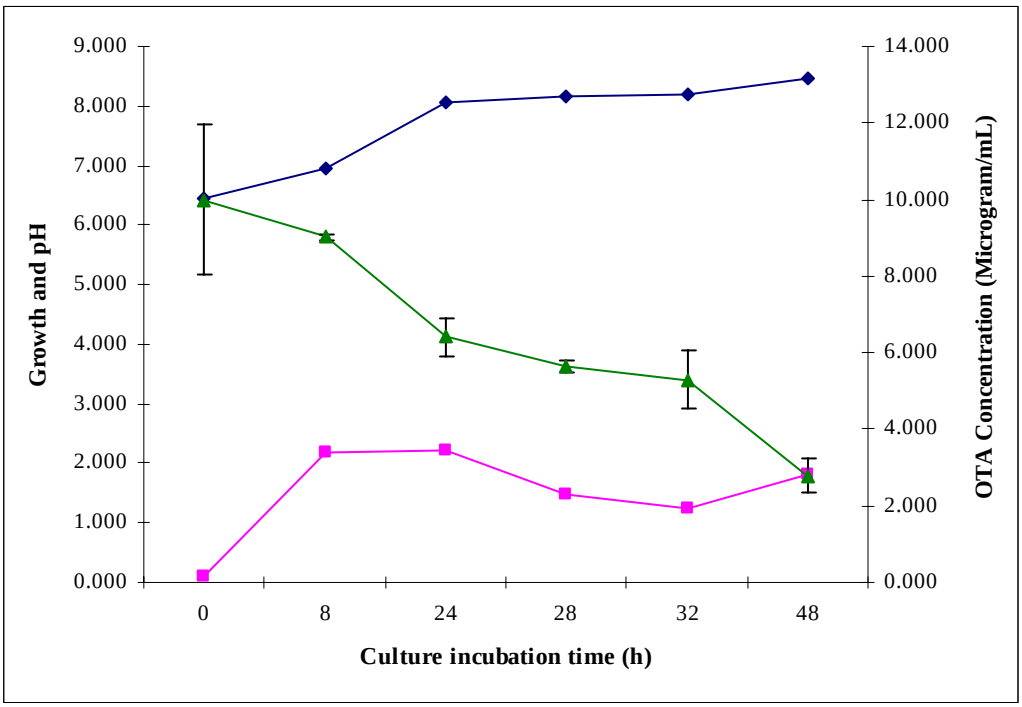

Fig. 5.4 Growth (■), pH (◆) and OTA kinetic degradation (▲) by culture supernatant from *Bacillus* MHS 13 at pH 5 after 2 h contact time.

For this strain the optimal pH and the optimal culture time are different since the maximal activity was reached after 48 h of culture and the optimal is pH 5.0. This could explain why in the former part during screening of *Bacillus* for OTA

degradation activity (cf chapter IV) a very weak OTA decrease could be noted during culture of this strain since the culture medium had a pH greater than 7 since the first hours.

Regarding to AFB_1 removal by culture supernatant from both *Bacillus* CM 21 and *Bacillus* MHS 13, results showed that neither culture supernatant from both these selected strains could be degraded aflatoxin B_1 (data not shown).

Similarities between OTA degradation kinetics by *Aspergillus niger* (Abrunhosa et al., 2006) and our *Bacillus* isolates and the detection of the degradation product ochratoxin α in the fermented broth of *Bacillus* isolates suggested that a carboxypeptidase A activity may be responsible for OTA decomposition by these isolates. OTA is a competitive inhibitor of carboxypeptidase A. So, Carboxypeptidase A will cleavage OTA into OTα and phenylalanine. OTα is being commonly reported as less toxic than ochratoxin A. For example, ochratoxin α was ineffective as an immunosuppressor when tested in mice, unlike ochratoxin A or (4R)-4-hydroxyochratoxin A which reduced the production of immunoglobulin M and G from 80 to 93% (Creppy et al., 1983). Also, it was reported that ochratoxin α is, at least, 1000 times less toxic than ochratoxin A to brain cell cultures (Bruinink et al., 1998). Furthermore, ochratoxin α elimination half-life in the body (9.6 h.) is shorter then that of ochratoxin A (103 h.); so, treatments which enhanced the conversion of this mycotoxin into ochratoxin a are considered to be a way to reduce its toxicity (Li et al., 1997).

5.4.2 Degradation of AFB_1 by cellular extracts

In this part, study on AFB_1 degradation by cellular extract from both selected strains (*Bacillus* CM 21 and *Bacillus* MHS 13) were conducted. Results showed that their cellular extracts which were collected at any different cultures incubation time and at different cultures growth phase could not degrade AFB_1 (data not shown). Moreover, proteins precipitation by different concentrations of ammonium sulphate and then concentration by dialysis were also performed. Unfortunately, the precipitate concentrated by a factor 200 could not degrade AFB_1 (data not shown).

Accordingly to the results we presented above, this means that enzymatic degradation is not involved in AFB_1 degradation by both *Bacillus*. However, during the growth of whole cells of *B. subtilis* MHS 13 and *B. licheniformis* CM 21, a percentage of AFB_1 decrease of respectively 74% and 85% had been observed. Consequently, there are two possible mechanisms which will be involved. First, the degradation mechanism may be due to ammoniation since for both strains could be able to produce high ammonium concentrations were reached at the end of the culture (table 5.1). The detail of this mechanism, we had already mentioned in chapter IV.

The other one possible mechanism which is also our hypothesis could be cell adsorption at cell wall (peptidoglycan). Because of *Bacillus* spp. are Gram-positive bacteria as same as *Lactobacillus* spp. The cell wall of *Bacillus* is a rigid structure on the outside of the cell that forms the first barrier between the bacterium and the environment, and at the same time maintains cell shape and withstands the pressure generated by the cell's turgor. The cell wall is composed of peptidoglycan, teichoic and teichuronic acids as same as the cell wall of LAB. In brief, LAB cell wall consists of the peptidoglycan matrix forming major structural component of cell wall housing

other components such as teichonic and lipoteichoic acid, proteinatious S layer and neutral polysaccharides. These components play various functions including adhesion and macromolecule binding, especially fibrillar network of teichoic acids and neutral polysaccharides (Shetty and Jespersen, 2006). Thus, the peptidoglycan of Gram-positive is thick and thicker than the peptidoglycan of Gram-negative bacteria. Figure 5.5 showed the characteristic of cell wall of Gram-positive and Gram-negative bacteria.

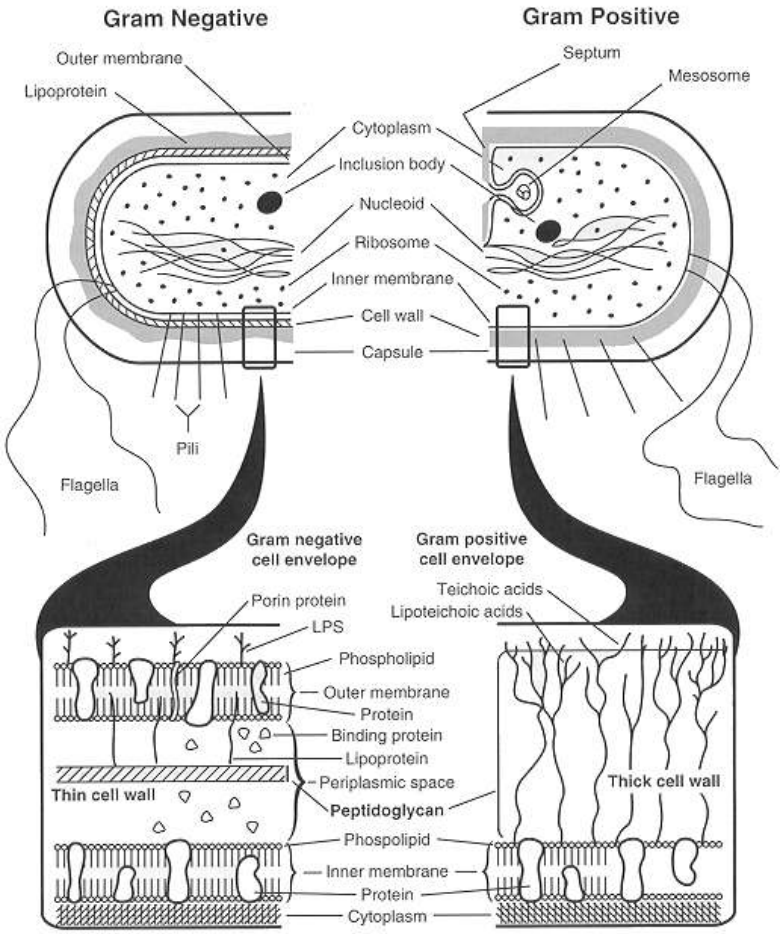

Fig. 5.5 Cell wall characteristic of Gram-positive and Gram-negative bacteria.

Theoretical calculations by Oatley et al. (2000) demonstrate that AFB_1 removal does not arise solely from trapping of the toxin in the bacterial pellet during

centrifugation. Metabolic conversion and covalent binding of AFB_1 by the bacteria have been excluded as a mechanism of removal, and non-covalent binding of AFB_1 from the bacteria has been proposed and nonviable bacteria also have high binding ability and more than viable bacteria. On the contrary with viable *Flavobacterium aurantiacum* AFB_1 removal from solution was irreversible by metabolic degradation following the formation of a loose complex with the periphery of the bacteria (Ciegler et al. 1966).

Moreover, Haskard et al. (2001) showed that surface components of the bacteria are involved in binding and binding of aflatoxin B_1 appears to be predominantly extracellular for viable cell and do not possess an S-layer but rather are encompassed by a polysaccharide capsule during growth. Besides, binding is of a reversible nature, but the stability of the complexes formed depends on strain, treatment, and environmental conditions. They also suggested that AFB_1 is bound to the bacteria by weak non-covalent interactions, such as associating with hydrophobic pockets on the bacterial surface. Haskard et al. (2000) found that the hydrophobicity of the AFB_1 molecule most closely matches that of chloroform. So, hydrophobic interactions play a major role in the binding mechanism.

Heat and acid treatments also significantly enhanced the ability of bacteria to remove AFB_1, with acid treatment being more effective than heat treatment in most cases. However, other studies (Haskard et al., 1998) have shown that the relative amounts of AFB_1 removed by viable and heat- and acid-treated bacteria depend on initial AFB_1 concentrations. Cell wall polysaccharide and peptidoglycan (Morotomi et al., 1986) are the two main elements responsible for the binding of mutagens. Both of these components are expected to be greatly affected by heat and acid treatments.

Heat may cause protein denaturation or the formation of maillard reaction products between polysaccharides and peptides or proteins. Acid may break the glycosidic linkages in polysaccharides, releasing monomers that may then be further fragmented into aldehydes. Acid may also break the amide linkages in peptides or proteins to produce peptides and the component amino acids. Hence, acid treatment may break down the peptidoglycan structure, resulting in the observed compromised structural integrity. Figure 5.6 showed the structure of both Gram-positive and Gram-negative peptidoglycan.

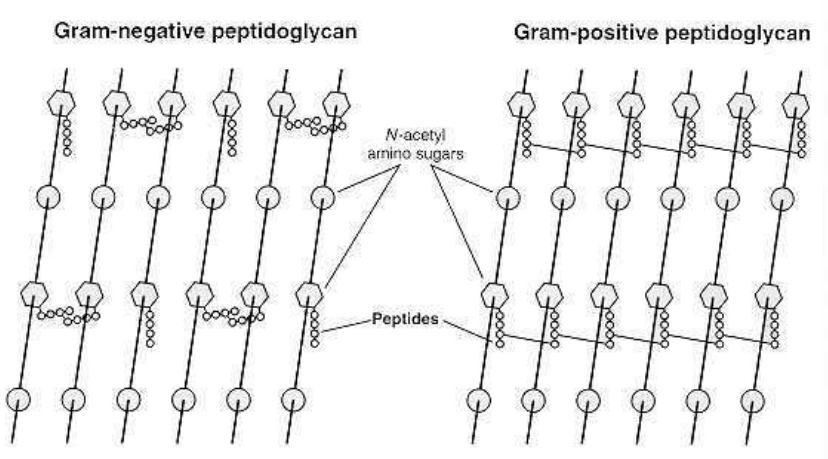

Fig. 5.6 Structure of peptidoglycan of Gram-positive and Gram-negative bacteria.

Although the peptidoglycan layer is quite thick in these organisms, there may be a decrease in thickness, reduction in cross-links, and/or increase in pore size. This perturbation of the bacterial cell wall may allow AFB_1 to bind to cell wall and plasma membrane constituents that are not available when the bacterial cell is intact. The effective removal of AFB_1 by all nonviable bacteria suggests that binding rather than metabolism is involved in all cases. Finally, the involved mechanism in our case

could not be elucidated here and still remains unknown. It should be investigated in future studies.

The observation that *Bacillus* isolate is able to degrade OTA is promising because it might allow the biological elimination of this mycotoxin. These bacteria provide a source of enzymes which could be used for detoxification of OTA in contaminated agricultural products.

5.5 Conclusion

From this study, it appears that AFB_1 could be degraded by neither culture supernatant nor cellular extract from these microorganisms. Moreover, no degradation product of AFB_1 could be detected by HPLC analysis either during AFB_1 removal by whole cells of *Bacillus* or during their incubation in nutrient broth. So the mechanism remains unknown. Perhaps, it will be due to the chemical reaction that is ammoniation or cell adsorption.

In the case of OTA detoxification, we observed that it is also efficiently detoxified by some *Bacillus* isolates and especially by *B. licheniformis* CM 21. The degradation product, ochratoxin α, in the fermented broth of *B. licheniformis* (*Bacillus* CM 21) had been detected. Thus, carboxypeptidase A may be responsible for OTA decomposition by these isolates.

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CHAPTER VI

CONCLUSION AND PERSPECTIVES

Thua-Nao is a vegetable protein and also is one of the oldest traditionally fermented soybean products which is widely consumed as a substitute for fish sauce by most of Thai people who lives in the Northern of Thailand. The soybeans are fermented with *Bacillus* spp. after soaking and cooking to yield a protein-rich food.

However, soybean could be contaminated by some mycotoxins during post-harvest and/or storage condition particularly by aflatoxin B_1 (AFB_1) and ochratoxin A (OTA). These mycotoxins are classified into group 1A and 2B by IARC respectively owing to their degrees of toxicities to human and domestic animals.

Fortunately, *Bacillus* spp. which is the dominant microflora of this product can exhibit a potent mycotoxin (AFB_1 and OTA) detoxification ability. We showed that among 23 isolates from fresh Thua-Nao, 12 were able to inhibit growth of *A. flavus* and 8 the growth of *A. westerdijkiae*. In the same time half of the isolates decreased the aflatoxin level of at least 60% and 6 of them decreased the OTA level of at least 50%.

In our work we specially focused on 2 isolates: one *Bacillus licheniformis* and one *Bacillus subtilis*. These microorganisms showed an OTA-degradation activity. The OTA decrease was due to the degradation in ochratoxin alpha (OTα) and the degradation mechanism involved was shown to be extracellular. Culture supernatant from *Bacillus licheniformis* has the highest percentage of OTA degradation (97.5%), greater than culture supernatant from *Bacillus subtilis* (69%) in nutrient broth (NB). Optimum activity of culture supernatant from *Bacillus licheniformis* was found at pH

7.0 and 37°C with 24 hours culture incubation time and 2 hours contact time, whereas for *B. subtilis* the optimal conditions for extracellular activity were pH 5.0, 37°C and 48 hours of culture.

On the other hand, AFB_1 removal could be observed during culture of both *Bacillus* strains but the decrease had not been correlated to appearance of a degradation product. Moreover, AFB_1 could be degraded by neither culture supernatant nor cellular extract from these microorganisms. The possibility of AFB_1 reduction exhibited by whole cells which may be due to a non-enzymatic mechanism.

Hence, decontamination of mycotoxins using microorganisms is one of the well-known strategies for the management of mycotoxin in food and feed. Although there are many different approaches available for mycotoxin decontamination it seems the most interesting since the microorganisms can belong to the natural flora of the product. However, questions remain on the toxicity of products of enzymatic degradation and undesired effects of fermentation in case of non-native microorganisms on quality of food. Another advantage of biological decontamination is that co-contamination by 2 toxins can be decreased in one step choosing the adequate strain.

Thus, Thua-Nao can be safe for consumer. Furthermore, *Bacillus licheniformis* and *Bacillus subtilis* could be a source of an efficient enzyme for OTA degradation in other food and/or feeds and also could be a potential control of fermented soybean product quality.

Finally, further studies should be performed on: the effect of time and temperature during soybean fermentation on mycotoxin reduction, characterization of extracellular activity of OTA degradation and the conditions of use of selected *Bacillus* starters in order to monitor mycotoxins level in Thua-Nao.

BIOGRAPHY

Awanwee Petchkongkaew was born in Bangkok, Thailand. She attended Thammasat University, Thailand and received her Bachelor's degree in Food Science and Technology (1998). In 2001, she received a Master's degree in Environmental Sanitation at Mahidol University, Thailand and in 2002 she was granted a scholarship by NSTDA jointly with Thammasat University to study her Ph.D program at School of Food Technology, Suranaree University of Technology. During study her Ph.D. Program, in 2005, she participated a joint scientific and administrative supervision of doctoral thesis between Suranaree University of Technology (SUT, Nakhon Ratchasima, Thailand) and L'Institut National Polytechnique de Toulouse (I.N.P. Toulouse, France) which supported by the grants from The Franco-Thai Cooperation in Higher Education and Research in Tropical Agronomy and Agro Industry.

Poster presentation:

1. Awanwee Petchkongkaew, Patricia TAILLANDIER, Piyawan Gasaluck and Ahmed LEBRIHI. 2005. Interaction between *Bacillus subtilis* and *Aspergillus flavus* Isolated from Thai Fermented Soybean Product. *Euro-Maghrebin Symposium on Biological, Chemical Contaminants and Safety in Food, September 7-9, 2005, Fez, MOROCCO.*

2. Awanwee Petchkongkaew, Ahmed LEBRIHI, Piyawan Gasaluck and Patricia TAILLANDIER. 2007. Reduction of Mycotoxin by *Bacillus* spp. Isolated from Fermented Soybean. *Food Innovation Asia 2007 "Q" Food for Good Life, June 14-15, 2007, BITEC Bangna, Bangkok, THAILAND.*

Publication

1. Awanwee Petchkongkaew, Patricia TAILLANDIER, Piyawan Gasaluck, and Ahmed LEBRIHI. Isolation of *Bacillus* spp. from Thai fermented soybean (Thua-nao): screening for aflatoxin B_1 and ochratoxin A detoxification. *Article in press. Journal of Applied Microbiology.*